

**Advanced steam-explosion pretreatment mediated anaerobic digestion of municipal sludge:
Effects on methane yield, emerging contaminants removal, and microbial community**

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23 Graphical Abstract

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Dewatered Sludge [TS=20%]



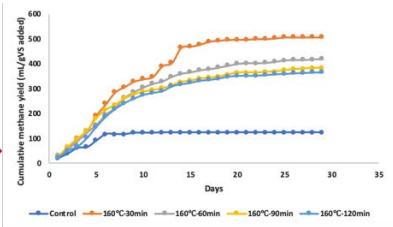
Thermal Hydrolysis Reactor
TS = 14.0%

Pretreatment conditions	
120°C-	30,60, 90,120min
140°C-	30,60, 90,120min
160°C-	30,60, 90,120min
180°C-	30,60, 90,120min



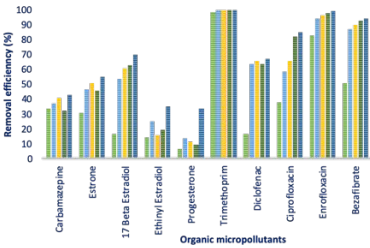
BMP Assay (35°C)

Bio-methane
Potential &
Kinetic modelling



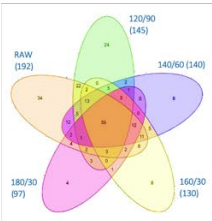
- Thermal hydrolysis enhanced organics solubilization by 40% over control.
- Highest Methane yield at 160°C, 30min- 506 mL CH₄/g VS_{added}

Organic
Micropollutants
Removal



- Effective removal of micropollutants achieved except carbamazepine, progesterone, and 17α- ethinylestradiol
- Highest removal (>80%) -Trimethoprim, Enrofloxacin and Bezafibrate

Metagenomic Study



- Unique and shared OTUs among variably THP pretreated and digested sludge samples

➤ THP-mediated anaerobic digestion of sludge shows better performance than the control sludge, improving methane yield, higher VS and micropollutants removal, and a diverse microbiome in the digester.

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Abstract

Advanced steam explosion pretreatment, i.e., the Thermal hydrolysis process (THP) is applied mainly to improve the sludge solubilization and subsequent methane yield in the downstream anaerobic digestion (AD) process. However, the potential of THP in pretreating the high solids retention time (SRT) sludges, mitigating the risk of emerging organic micropollutants and effects on anaerobic microbiome in digester remains unclear. In this study, sludge from a sequencing batch reactor (SBR) system operating at a SRT of 40 days was subjected to THP using a 5L pilot plant at the temperature ranges of 120- 180°C for 30- 120 min. The effect of THP on organics solubilization, methane yield, organic micropollutant removal, and microbial community dynamics was studied. The highest methane yield of 507 mL CH₄/g VS_{added} and volatile solids (VS) removal of 54% were observed at 160°C- 30min THP condition, i.e., 4.1 and 2.6 times higher than the control (123 mL CH₄/gVS_{added}, 20.7%), respectively. The experimental values of hydrolysis coefficient and methane yield have been predicted using Modified Gompertz, First order, and Logistics models. The observed values fitted well with all three models showing an R² value between 0.96 to 1.0. THP pretreated sludges showed >80% removal of Trimethoprim, Enrofloxacin, Ciprofloxacin, and Bezafibrate. However, Carbamazepine, 17 α -ethinylestradiol, and Progesterone showed recalcitrant behavior, resulting in less than 50% removal. Microbial diversity analysis showed the dominance of *Proteobacteria*, *Firmicutes*, *Chloroflexi*, and *Bacteroidetes*, collectively accounting for > 70-80% of bacterial reads. They are mainly responsible for the fermentation of complex biomolecules like polysaccharides, proteins, and lipids. The THP-mediated anaerobic digestion of sludge shows better performance than the control digestion, improved methane yield, higher VS and micropollutants removal, and a diverse microbiome in the digester.

Keywords: Thermal hydrolysis process (THP), sewage sludge, bio-methane, micropollutants

1. Introduction

Anaerobic digestion (AD) is a robust process to stabilize the sludge, reduce the sludge volume, and recover renewable energy (Tyagi and Lo, 2013). However, the complex structure of sludge, i.e., intracellular and extracellular polymeric substances (EPS), limits its biodegradation resulting in a low digestion rate and solid reduction (Yan et al., 2022). Several sludge pretreatment techniques, such as thermal, chemical (alkaline), mechanical (ultrasonication), and biological pretreatment (enzymatic), have been developed to improve the bio-methanation of sludge (Morgan-Sagastume et al., 2011; Tyagi and Lo, 2013; Yan et al., 2022). Among them, the thermal hydrolysis process (THP) has been studied broadly and commercialized successfully (Barber, 2016; Gahlot et al., 2022; Yan et al., 2022). Under high temperatures and pressure, typically 165°C and 6 bar, microbial cell, and cellular polymer disintegration results in enhanced sludge solubilization (Barber, 2016; Jeong et al., 2019). The critical mechanisms involved are hydrolytic reaction, free radical oxidation, and sudden pressure drop, which result in cell disintegration. Previous studies have shown that temperature is the most influential parameter affecting sludge biodegradability and methane production (Barber et al., 2016). An increase in reaction temperature from 90°C to 100°C resulted in a 68.4% increase in methane production (Yan et al., 2013). Nevertheless, an increase in solubilization degree does not necessarily result in enhanced methane production owing to the formation of soluble refractory and inert compounds at higher temperatures, i.e., >160°C (Balasundaram et al., 2022). Earlier work revealed that the optimum temperature for pretreatment ranges from 160-180°C (Bougrier et al., 2008; Toutian et al., 2020).

Previous studies have focussed on the thermal hydrolysis pretreatment of sludge collected from conventional activated sludge process (CASP) based wastewater treatment Plant (WWTP). In relatively smaller communities, sequencing batch reactor (SBR) based WWTPs are operated at higher solids retention time (SRT), and the investigations on thermal hydrolysis of high SRT sludges and subsequent anaerobic digestion are limited. Another, less researched aspect of the thermal hydrolysis process is the fate of organic micropollutants (OMPs) in the context of municipal sludge. Organic micropollutants involve personal care products, pharmaceuticals, pesticides, steroid hormones, and industrial chemicals. The concentrations of these compounds may vary from some ng/ kg to g/kg dry weight basis (Stasinakis et al., 2012). Due to the analytical complexities, little attention has been given to analyzing OMPs in the sludge matrix. As pretreatment degenerates the complex polymeric sludge matrix, it is expected that OMPs originally encased within the sludge matrix, could be liberated into the soluble phase and offer them more readily for biodegradation due to the alterations in their molecular structures under variable pretreatment conditions, i.e., chemical dose, mechanical shear, high or low temperatures. Moreover, as sludge pretreatment solubilizes the organic fraction (likely OMPs), it may be hypothesized that there is a probability to switch the final disposal of remaining OMPs in digestate from farmland or landfill to the main wastewater stream (Hamid and Eskicioglu, 2013). Thus, this study also investigated the combined effects of thermal hydrolysis-mediated anaerobic digestion on the removal of OMPs in the context of SBR sludge. Also, there has been increased attention in the last decade to understanding the link between microbial community dynamics and the anaerobic digester performance under dynamic operational conditions. Exploring this link could help the operators provide more optimum conditions that favor the activity of the highest-performing microbial community affecting the process performance.

Thus, given the lack of research focused on the thermal hydrolysis of sludge in the context of the fate of micropollutants and microbial diversity in the digester, this work aims to determine the effect of thermal hydrolysis on 1) sludge solubilization and methane yield 2) removal of organic micropollutants, and 3) microbial community structure in the digester. This work highlighted the efficiency of the thermal hydrolysis process for the sludge collected from a sequencing batch reactor (SBR) system operating at a higher solids retention time (SRT) of 40 days.

2. Materials and methods

2.1. Substrate and inoculum

Sewage sludge for batch assays was obtained from a municipal wastewater treatment plant (WWTP), based on a sequencing batch reactor (SBR) process, at the Indian Institute of Technology (IIT) Roorkee, Uttarakhand, India. The SBR system operated at a higher solids retention time (SRT) of 40 days. Sewage sludge was freshly collected before thermal hydrolysis pretreatment, and batch assays were carried out. The physical-chemical characteristics of sludge have been summarized as pH 7.20, TS 14%, VS 54% (of TS), TKN 2.2%, $\text{NH}_4^+\text{-N}$ 290 mg/L, tCOD 106000 mg/L, sCOD 7536 mg/L, VFA 600 mg HAc/L, total protein 17.12 g/L, soluble protein 2346 mg/L, and total alkalinity 2800 mg CaCO_3 /L. The inoculum was procured from a digester treating primary and secondary sludges mix at an activated sludge process-based municipal WWTP located at Jagjeetpur, Haridwar, India. The inoculum was allowed to settle for two hours to drain the excess water. The inoculum was then transferred to 1L glass bottles and allowed for degassing and acclimatization for seven days by keeping at 35°C in an orbital shaker incubator (RIS-24 PLUS,

REMI, India). The pH of the inoculum was measured once every two days using a pH meter (TOSCHON, Toshniwal, India).

2.2. Thermal hydrolysis pretreatment

Thermal hydrolysis pretreatment was carried out using a Mini steam explosion unit with a reactor volume of 5L (CAMBI Group AS, Norway). The pilot plant includes a steam generator, a reactor for pretreating sludge, and a flash tank. Dewatered SBR sludge (14% TS) was pre-treated at variable temperatures-pressures (120°C-2 bar, 140°C-3.5 bar, 160°C-6 bar, and 180°C-10 bar) and pretreatment time (30- 60- 90- 120 min) using steam injection into the THP reactor. After the desired reaction time, the sludge was transferred to a flash tank resulting in enhanced cell disintegration from the rapid pressure drop. The sludge was cooled down at room temperature in closed containers before being used for batch assays.

2.3. Biomethane potential (BMP) assays

The biomethane potential (BMP) assays were carried out using amber glass bottles of 350 mL working volume (SUPELCO Analytical, Sigma, USA). The acclimatized inoculum was mixed with the substrate (sewage sludge) by maintaining the inoculum-to-substrate (ISR) ratio of 1:1 (volatile solids, VS basis). Batch assays were conducted in duplicates. After adding inoculum and substrate in the required proportion, N₂ gas was purged into the assay bottles for 2 min to remove oxygen and to support the anaerobic state. The assay bottles were immediately capped and put in an incubator shaker at 80 revolutions per minute (rpm) and 35°C. Daily biogas production was observed through an HHP350 smart manometer (Omega, USA). Biogas pressure was measured in the bar and converted into the volume by employing the ideal gas law Eq. 1. (Ahmed et al., 2022)

$$V = (Pr.Ta)V_r/(Pa.Tr) \quad [1]$$

Where, V = volume of daily biogas produced (mL). T_a = Ambient temperature (K). P_r = pressure in the reactor (kPa). P_a = ambient pressure (kPa). V_r = headspace volume (mL), T_r = temperature of reactor (K).

The biogas generated in the blank assay (inoculum only) was deducted from the biogas produced in the test assays (inoculum+ substrate). The pH of the assays was measured every day and corrected to 7.5-8.0 by adding 1N-NaOH solution if the pH dropped below 6.5. The experimental run was stopped once the daily biogas generation rate approached a minimum, i.e., < 2mL/day.

2.4. Analytical methods

The control (raw) and thermally hydrolyzed sludge samples were examined for various physical-chemical parameters, i.e., total solids (TS), total Kjeldahl nitrogen (TKN), volatile solids (VS), chemical oxygen demand (total tCOD, soluble sCOD), pH, and proteins (total tProteins, soluble sProteins). All the experiments were carried out according to Standard Methods (APHA, 2017). For analysis of NH₄⁺-N and volatile fatty acids (VFA), the sludge samples were diluted (100x) with Milli-Q water and then centrifuged at 8000 revolutions per minute (rpm) (4500 relative centrifugal force, rcf) for 10min. The supernatant was filtered using 0.45µm glass fiber filter paper, and the filtrate was used for analysis (Ahmed et al., 2022). The Nessler method (HACH 8075) was followed to determine TKN using the UV-VIS spectrophotometer (HACH DR6000). For VFA determination, the filtrate was titrated with 0.1N- or 0.05N-NaOH. Finally, the titrant volume was used to calculate the VFA concentration (DiLallo and Albertson, 1961). The total and soluble proteins were analyzed by the Lowry assay (Lowry et al., 1951) using the spectrophotometer at

620nm wavelength. Analysis of the parameters was carried out in replicates and an average of the values has been reported along with the standard deviation. Biogas composition was measured using a Geotech Biogas 5000 analyzer (QED Environmental Systems Ltd, UK). The solubilization of COD was measured as per the Eq.2 (Gong et al., 2019),

$$\text{Solubilization of COD} = \frac{sCOD_2 - sCOD_1}{tCOD_1 - sCOD_1} \times 100\% \quad [2]$$

Where, $sCOD_1$ = sCOD of non pre-treated sample; $sCOD_2$ = sCOD of pre-treated sample; $tCOD_1$ = tCOD of non pre-treated sample.

2.4.1. Organic micropollutants detection

The fate of ten organic micropollutants, i.e., one anti-epileptic (Carbamazepine), one antiphlogistic (Diclofenac), three oestrogens (estrone (E1), 17β -estradiol (E2), 17α -ethinylestradiol (EE2), one hormone (progesterone), three antibiotics (Trimethoprim, Ciprofloxacin, Enrofloxacin), one anti-lipemic (Bezafibrate) were studied in anaerobic digestion of THP sludge. The dewatered sludge samples were lyophilized to perform the ultrasonic solvent extraction. After lyophilization, the samples were ground using a mortar and pestle. Two sequential extractions of a 2.0 g freeze-dried sample were performed using 15 mL of each acetonitrile and phosphate buffer (pH 2). During the first two extractions, the samples were sonicated for 30 min and centrifuged for 5 min at 3000 rpm. The samples were vortexed for 5 min before each sonication. The third extraction was performed similarly to the first two extractions, but without adding phosphate buffer. The tubes were sonicated, centrifuged, and decanted. All the decanted supernatant were combined and evaporated at 40°C to a final volume of 20 mL. The samples were re-suspended by adding 200 mL of distilled water, followed by filtration using Polytetrafluoroethylene (PTFE) filter paper, 0.45µm. The filtered samples were then extracted using the solid phase extraction (SPE) method.

2.4.1.1. Solid phase extraction method

The sample extraction was carried out as per Biswas et al., (2021) using 200 mg OASIS HLB cartridges. The cartridges (Waters, Milford, MA, USA) were first conditioned with 6 mL methanol and later with 6 mL deionized water. The acidified samples (pH = 2) of 200 mL volume were passed through the cartridges (1-2 drops/sec). The cartridges were dried under vacuum for 15 min, followed by a series of eluents. The first elution was performed using 8 mL of acidified methanol (0.1% acetic acid v/v) followed by 6 mL of 50:50 acetonitrile and isopropanol solution. The subsequent elution was performed using 3 mL each of ethyl acetate followed by hexane. The eluents were dried under a gentle nitrogen flow and reconditioned to 2 mL with a 90:10 water-methanol mix. Later, the samples were filtered using 0.2 µm filters and examined using HPLC-MS.

2.4.1.2 Method for HPLC-MS

HPLC-MS from Waters® has been used to quantify the target compounds in the extracted samples. The chromatographic separation was achieved using a reverse-phase column (C18, 4.6 X 250 mm) maintained at 40°C along with an optimized gradient program. The samples were kept at a temperature of 15°C, and a 10 µL injection volume was used. A 25 min gradient program was used along a continuous flow rate of 0.4 mL per minute. The program used water and 0.1% acetic acid (v/v) as mobile phase A, while methanol was used with 0.1% acetic acid (v/v) as mobile phase B. The gradient started with 90% of mobile phase A, which was reduced to 60% at 2 min. At 5 min, flow from mobile phase A was stopped, which was held up to 11 min. The flow from mobile phase A was gradually increased up to 100% at 21 min, which again reduced to 60% at 22 min. At 24 min, phase A was increased to the initial flow of 90%, which was held up to 25 min. All the

compounds were quantified with single ion monitoring (SIM) mode. All the compounds were detected in positive (+) ESI mode. Nitrogen was used as a nebulizing gas. The capillary voltage of 3 kV was used for all the target compounds, while the cone voltage was set based on the direct infusion of individual stock solutions of each target compound. The retention times and cone voltages of all the target compounds are given in the supplementary information (Table S1).

2.5. DNA extraction and microbial diversity analysis

After completion of batch assays, 5 mL sludge samples were collected for each assay showing the highest methane yield, i.e., 120°C-90 min, 140°C-60 min, 160°C-30 min, 180°C-30 min. DNA was isolated using the commercially available kit (DNeasy PowerSoil Pro Kits, Qiagen, Germany)(Liu et al., 2021). DNA extraction was followed by the quantification of extracted DNA using NanoDrop. The NanoDrop readings were checked for absorbance ratio at 260/280, and a ratio between ~1.8-2.0 was obtained for each sample to determine the DNA and RNA quality. 40ng of isolated DNA was used for 16s RNA sequencing using V3-V4 region primers of 16s gene, i.e., V34F:- 5' AGAGTTTGATGTTGGCTCAG3' (forward primer) and V34R:- 5' TTACCGCGGCMGCSGGCAC3' (reverse primer). The sequencing was carried out with an Illumina MiSeq platform using a 2x300PE v3 kit. To enable accurate investigation at the genus level, raw data quality examination was conducted using MULTIQ and FASTQC, accompanied by trimming adapters and low-quality reads by TRIMGALORE. The trimmed reads were further processed (merging paired-end reads, chimera removal, OTU richness determination, and evaluation amendment) using QIIME/ MOTHUR/ KRAKEN/ BRACKEN workflows. Final OTUs were taxonomically classified using nucleotide BLAST against the database derived from SILVA/ GREENGENES/ NCBI. Alpha diversity analysis was performed through different indices

like Chao1, Shannon, Simpson, and Fisher with the statistical method of T-test /ANOVA. Beta diversity was calculated based on various global methods, and a heatmap was generated based on the Whittaker beta diversity method using Euclidean distance measures. The heat map was composed at the Genus level along a comprehensive view mode of <1500 properties. The samples were assembled with the Ward cluster algorithm based on the Euclidean distance measure. Detailed insights into dominating bacterial communities at phylum and genus taxonomic levels were critically analyzed from control (raw) and all pretreated test conditions (120°C-90min, 140°C-60min, 160°C-30min, 180°C-30min).

2.6. Statistical analysis

The performance of THP pretreatment was analyzed statistically at varying temperatures and treatment time, employing various statistical tools. The cumulative methane production obtained experimentally was examined for the goodness of fit through different kinetic models- The first order (FO) model, the Modified Gompertz Model (MGM), and the Logistic model (LM). These models assist in finding the most efficient metabolic pathway under variable pretreatment conditions. The FO is based on the hypothesis that hydrolysis is the rate-limiting stage in the AD of the organic substrates, thus helping to estimate the hydrolysis rate constant. The LM follows the hypothesis that the methane production rate is proportional to the quantity of methane generated. It helps to assess the initial exponential enhancement in methane yield and stabilize it at a later stage. The modified Gompertz model helps to estimate the lag phase and the maximal methane generation rate. The FO, LM, and MGM equations are represented by equations 3, 4, and 5, respectively (Rahmani et al., 2022),

$$M= G*(1-\exp(-kt)) \quad [3]$$

Where M is the methane production (mL); M_0 is the maximum methane production potential (mL)
 k is the hydrolysis co-efficient (dimensionless)

$$G(t) = \frac{G}{1 + \exp\left(4 \cdot \frac{R_{\max}(\lambda - t)}{Y} + 2\right)} \quad [4]$$

$$G(t) = G \cdot \exp\left\{-\exp\left[\frac{R_{\max} \cdot e}{Y} \cdot (\lambda - t) + 1\right]\right\} \quad [5]$$

Where $G(t)$ is the accumulative methane generation (mL); $R_{\max}$ is the maximal methane generation rate (mL/d); G is the ultimate methane generation potential (mL); T is the time (d); and λ is the lag phase time (d).

The IBM-SPSS Statistics 27 software was used to study the non-linear least squares regression analysis, including k , $R_{\max}$, λ , and the ultimate methane generation potential. The goodness of fit R^2 (coefficient of determination) was examined using the SPSS software. The principal component analysis (PCA) was performed using Minitab 17 to realize the correlation among the pretreatment conditions and batch assay results.

3. Results and discussion

3.1. Impact of thermal hydrolysis process on sludge characteristics

Thermal hydrolysis was conducted at a temperature range of 120-180°C for 30 min-120 min. When the pretreatment temperature was increased from 120 to 180°C, the pH of the sludge decreased from 7.20 to 6.07, respectively (Table S2). Under high temperatures, organic acids are released from cellular biomass resulting in a pH decrease (Xue et al., 2015; Jeong et al., 2019; Bougrier et al., 2008). More steam injection requirement into the reactor to increase the temperature from 120 to 180°C resulted in the reduction of TS from 14.4% (control) to 7.1% (180°C).

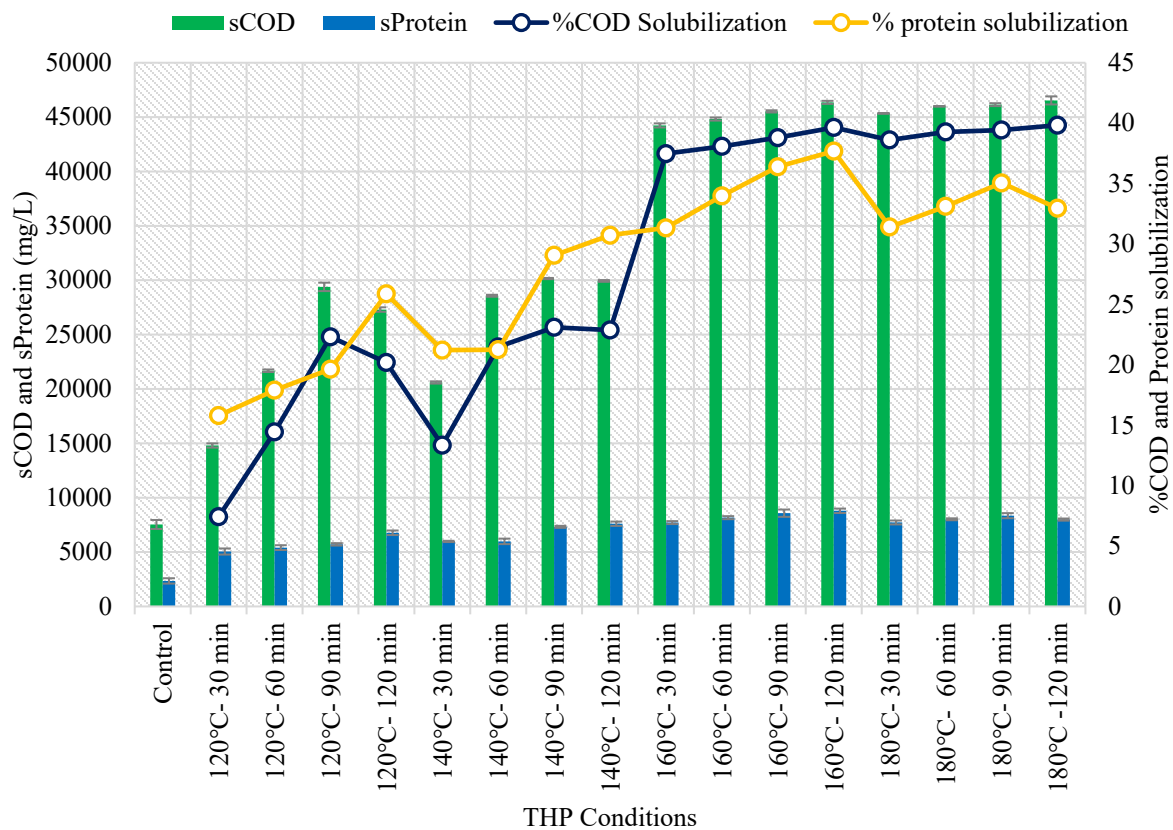
The efficiency of a pretreatment process can be determined by the increase in COD solubilization (Figure 1a). Thermal hydrolysis results in the disintegration of microbial cells and

particulate polymeric structure, followed by the release of soluble organics into the medium. Soluble monomers are formed from the polymers and then released into the liquid phase, which results in increased sCOD (Jeong et al., 2019). In this study, the thermal hydrolysis enhances the COD solubilization from 7% (120°C-30min) to 20% (120°C-120 min), 13% (140°C-30 min) to 23% (140°C-120 min), and up to 40% at 160°C. Further temperature increases beyond 160°C could not achieve any notable improvement in COD solubilization. Earlier studies reported that an increase in temperature increases sludge solubilization. The improvement was marginal from 100-120°C. However, a sharp increase was observed above 140°C (Jeong et al., 2019; Toutian et al., 2020). At moderate temperatures (100°C), microorganisms release small amounts of extracellular polymeric substances (EPS) that help to bond the gel structure in the sludge matrix. Above 140°C temperature, these gel-forming properties are lost, which results in higher solubilization at temperatures (Jeong et al., 2019).

Degradation of nitrogen-rich organics during THP could result in the increased concentration of ammonia nitrogen. Ammonia nitrogen exists as NH_3 and NH_4^+ in the liquid phase. The unionized ammonia form (NH_3) after entering the cell binds with the proton to form NH_4^+ which results in a pH imbalance inside the cell (Yan et al., 2022). Anaerobic digestion efficiency and methane generation can be affected at the high NH_4^+ -N concentration (2500-3500 mg/L) as it would impact the methane synthesizing enzymes (Chen et al., 2014; Higgins et al., 2017). In this study, NH_4^+ -N increased from 290 mg/L in the untreated sludge to a maximum of 596 mg/L at 180°C (Figure 1b). Under all studied temperatures, the reaction time of 30-60 min showed a slight increase in NH_4^+ -N. Once the treatment time exceeded 60min, NH_4^+ -N concentration increased gradually and doubled for a reaction time of 120 min. No significant increase in NH_4^+ -N was reported for a treatment time of 30min and temperature ranges of 120-180°C (Xue et al., 2015).

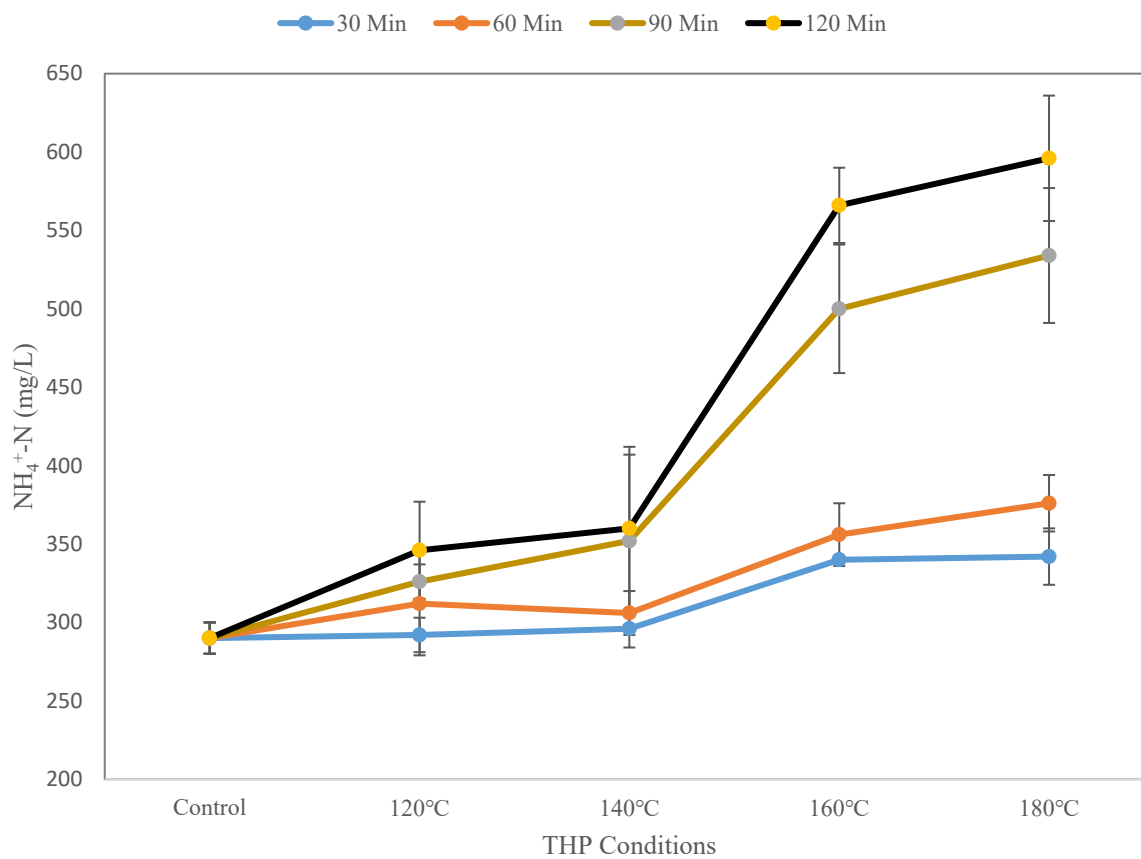
However, a sharp increase in $\text{NH}_4^+\text{-N}$ concentration was observed beyond 30min reaction time. Under high pretreatment temperatures (180-220°C), the $\text{NH}_4^+\text{-N}$ concentration could increase dramatically (Xue et al., 2015; Jeong et al., 2019).

The soluble protein concentration increased 2.7 times from 2300 mg/L in control sludge to a maximum of 8900 mg/L under the studied pretreatment conditions (Figure 1a). During thermal pretreatment, the proteinaceous fraction is solubilized as the hydrophobic groups in proteins are exposed. Moreover, the hydrophobic proteins are enclosed irreversibly through the gel produced by proteins under high-pressure conditions (Ahmed et al., 2022). Figure 1c shows that the temperature increase also enhances the VFA concentration from 600 mg HAc/L (control) to 2100 mg HAc/L owing to the effective hydrolysis of unsaturated lipids (Jeong et al., 2019). Figure 1 (a-c) shows the effects of the thermal hydrolysis process on the physico-chemical parameters of control and THP sludge.

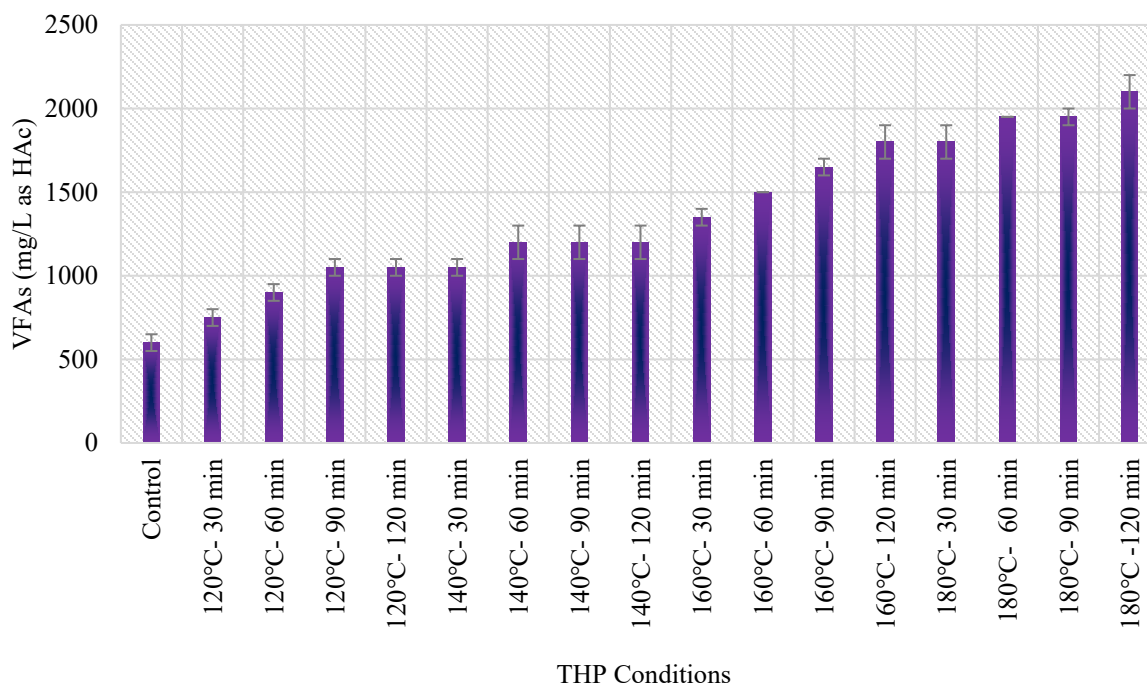


314

315 (a)



(b)



(c)

Fig. 1. Effect of thermal sludge hydrolysis on a) COD and protein solubilization b) $\text{NH}_4^+\text{-N}$ concentration and c) VFA profile

3.2 Effect of thermal hydrolysis process on biomethanation

3.2.1. Methane production

The biomethane potential (BMP) test is performed to evaluate the biogas generation potential of sludge through the conversion into methane. For a temperature of 120°C and variable reaction times, a maximum methane yield of $325 \text{ mL CH}_4/\text{g VS}_{\text{added}}$ was observed at 120°C -90min, i.e., 2.7 times higher than the control. The methane yield was reduced drastically to $167 \text{ mL CH}_4/\text{g VS}_{\text{added}}$ above 90min reaction times. At longer pretreatment times, the polymerization of low molecular amino acids and carbohydrates results in the formation of Amadori compounds, which could inhibit anaerobic digestion (Ngo et al., 2021). At a higher pretreatment temperature of 140°C - 90 min, the methane yield was increased by 2.4 times over the control. Among all the studied THP conditions, the highest cumulative yield of $507 \text{ mL CH}_4/\text{g VS}_{\text{added}}$ was achieved at 160°C -30min. An increased methane generation shows the abundance of rapidly degradable soluble organics fraction in the medium. It could be due to the intensive disintegration of the bacterial cell and EPS matrix under thermal hydrolysis resulting in the release of complex organic molecules, including carbohydrates, proteins, lipids, and nucleic acids. These molecules are then utilized by extracellular enzymes produced by anaerobes, resulting in enhanced methane production (Wang et al., 2009; Barber, 2016; Yan et al., 2022). During thermal hydrolysis, the reactivity of the water can be increased, which subsequently increases its rate of reaction with polymers resulting in compounds with smaller molecular weight (Eq. 6). These compounds can

then be easily accessed by the microbes and result in enhanced biomethane production. Also, increased temperature and oxygen-deficient conditions generate hydrogen and alkyl radicals.

During thermal hydrolysis, more reactive radicals such as, e.g., HO·, R·, and R₂N· are generated to oxidize organics inside the pre-treatment vessel. Further, carboxylic acids and short-chain alcohols are formed because of the reaction between free radicals. These compounds become the carbon source for anaerobes. The most crucial phase is the depressurization phase, in which shear force is applied to the cells, which further increases the sludge solubilization resulting in increased methane generation in downstream AD (Yan et al., 2022).



Above 160°C, the cumulative methane yield of the samples pretreated at 180°C decreased to 358 mL CH₄/g VS_{added}. Increasing pretreatment temperature and corresponding sludge solubilization may not always increase methane yield. Above a temperature of 160°C, simple sugars react with amino acids, resulting in Maillard reactions causing the formation of melanoidins (Jeong et al., 2019). Melanoidin formation occurs via three steps. In the first step, the amino groups present in the proteins and the carbonyl groups present in the sugars react together. This results in the formation of N- substituted glycosylamine which further forms 1-amino-1-deoxy- 2-Ketose due to amadori rearrangement. In the second step, yellow-coloured products are formed because of the dehydration and fragmentation of the products formed in the previous step. In the third step, condensation of the intermediates results in the formation of brown-coloured compounds called melanoidins (Yan et al., 2022). A similar trend of decrease in methane generation was reported once the pretreatment temperature was increased to 180°C (Xue et al., 2015). The effect of thermal hydrolysis pretreatment on cumulative methane yield is given in Figure 2. The normalized biogas composition is given in Figure S1. Due to the rapid decomposition of VFAs in the initial days, the

methane percentage in biogas composition showed an increasing trend. However, it became stable at 58-64% for the control assay. Meanwhile, Carbon-dioxide percentage in control ranged from 36-42%. Under stable conditions, THP sludge showed a methane concentration of 64%-78% and a carbon-dioxide concentration of 21-33%. It revealed that the pretreatment positively affected methane percentage in biogas because of altered sludge properties. These findings align with the study by Gong et al. (2019), who reported that the thermally pretreated sludge showed a higher methane percentage in biogas than the control sludge. Comparing the results of this study with previously reported studies, the highest cumulative methane yield of 507 mL CH₄/g VS_{added} is comparable with the values reported by Neumann et al., (2017) who stated that their cumulative methane yield values ranged between 472 and 611 mL CH₄/g VS_{added} for sewage sludge under varying pretreatment conditions. Higgins et al., (2017) stated that six fold differences were observed in methane production between the literature for almost similar pretreatment conditions (170-175°C) which could be due to the differences in sludge characteristics, testing conditions, solids content, heating and cooling periods ,etc.,

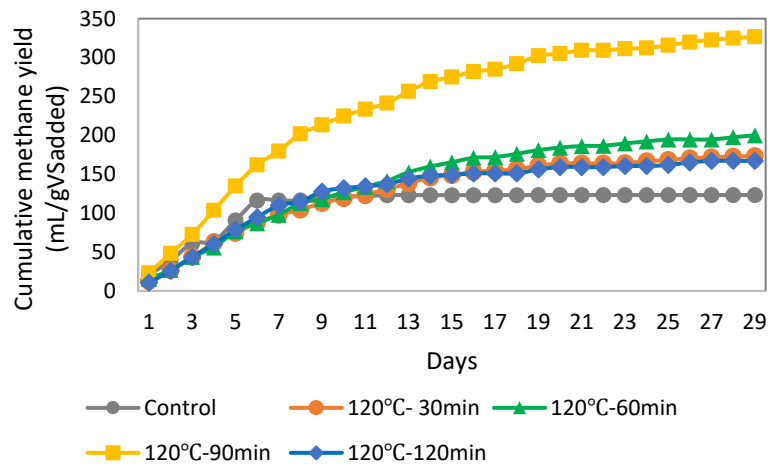
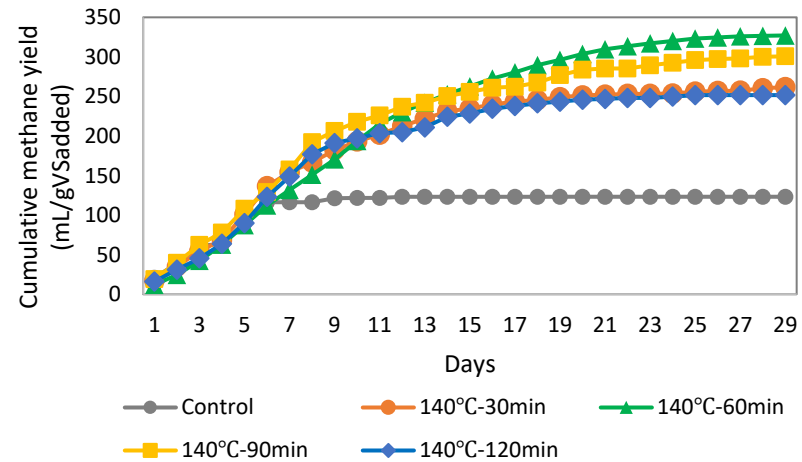
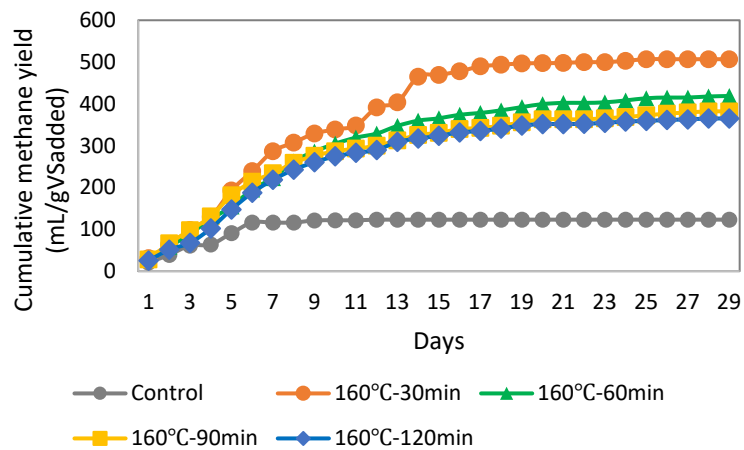
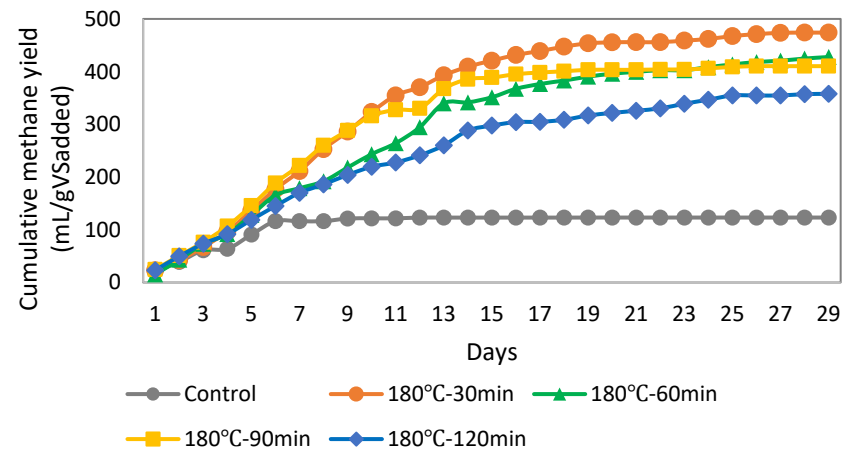
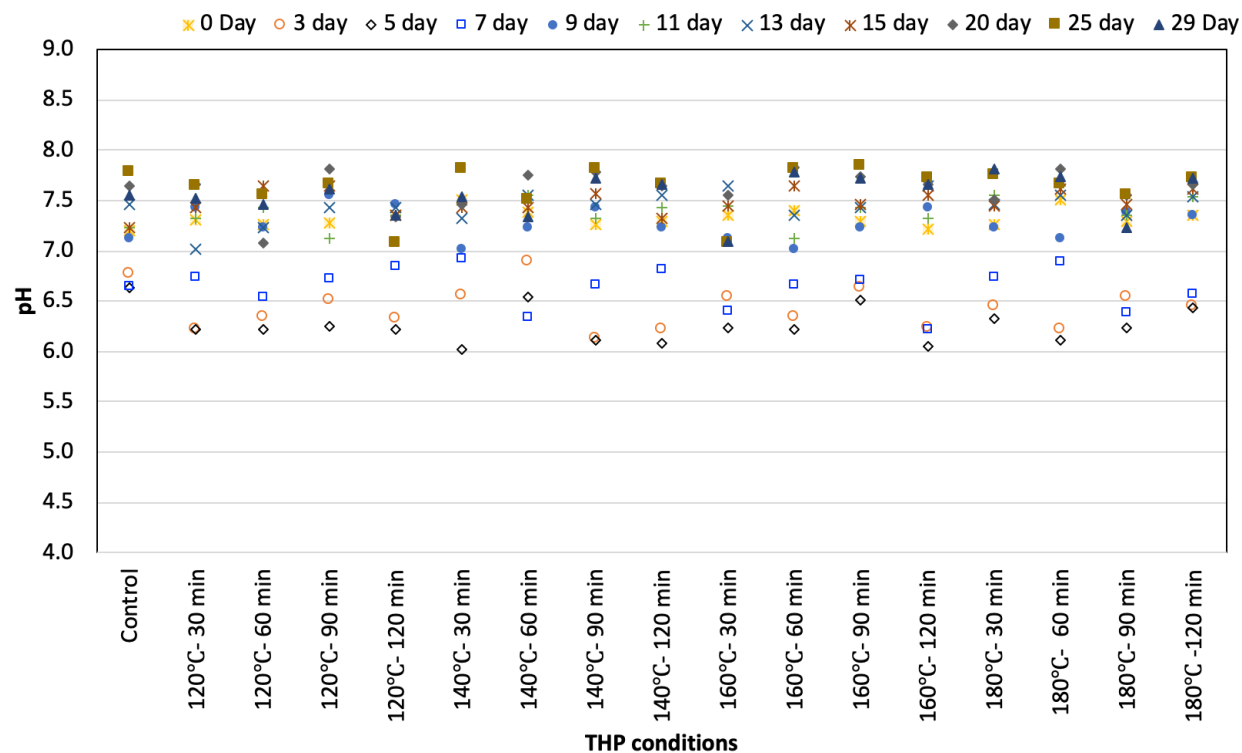


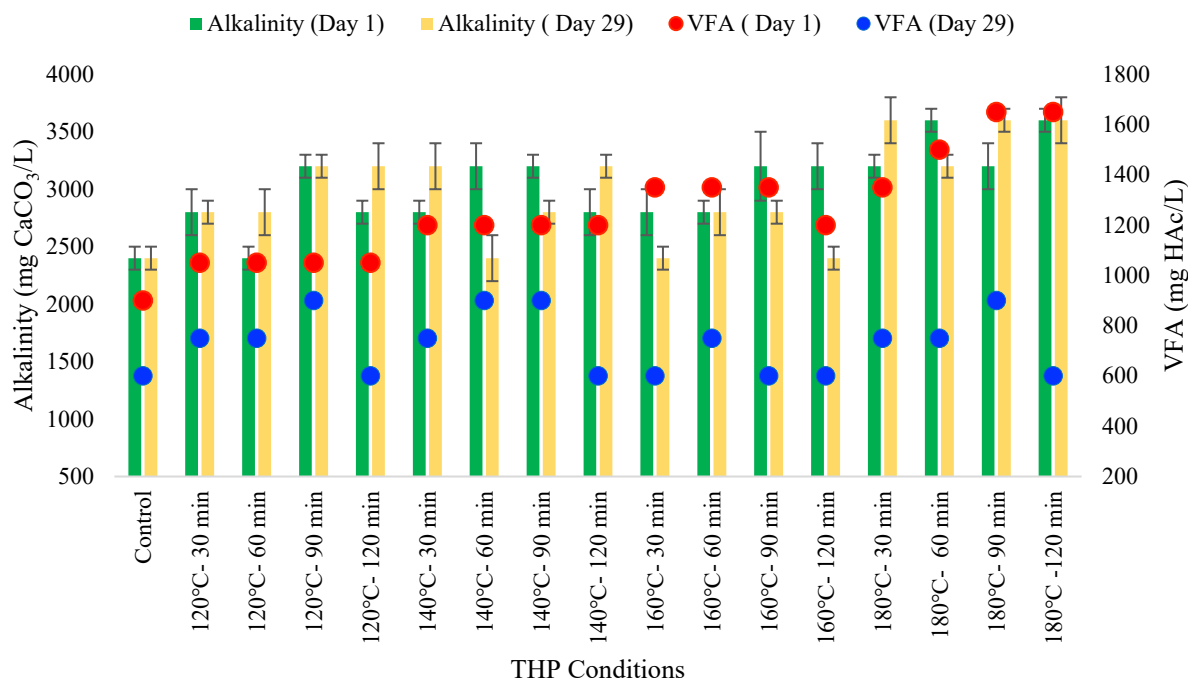
Fig. 2. Effect of thermal hydrolysis pretreatment on cumulative methane yield a) 120°C b) 140°C c) 160°C d) 180°C

3.2.2. pH, VFA, ammonia nitrogen and alkalinity

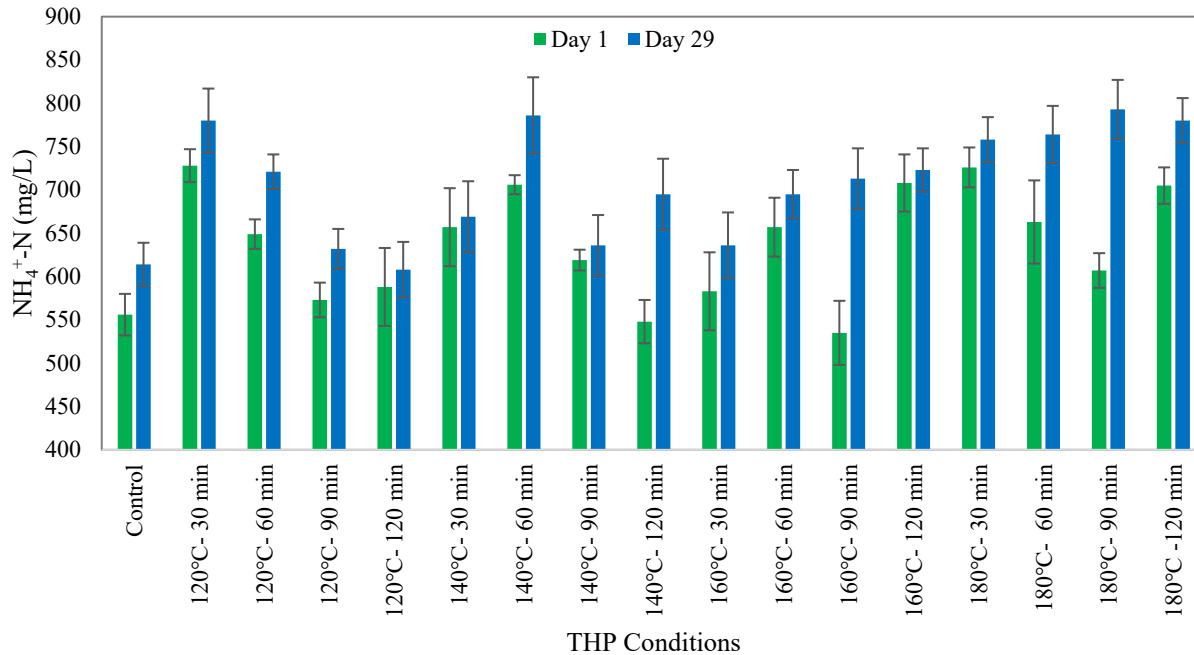
The pH of the BMP assays was monitored every day until stable values were reached. During the first week (Day 1-7), the pH values decreased to <6.5 owing to faster degradation of soluble organics and resulting a production (Figure 3a). The pH was maintained at 7.5 by adding 0.1N NaOH solution. The pH values were resumed to >7.0 after day 7 and no pH adjustment was required afterward. Similarly, an increase in VFA concentration was observed during the initial 5 days of the BMP assay owing to the high rate of acidogenesis (Figure 3b). The VFA concentration reached a maximum of 1950 mg HAc/L on Day 5, which decreased pH values. As compared to day 1, NH_4^+ -N concentrations were found to increase in all the assays during the study period. The NH_4^+ -N concentration reached a maximum of 780 mg/L among the different BMP assays (Figure 3c). The NH_4^+ -N concentration was less than the process-inhibiting range of $<1700\text{mg/L}$ (Chen et al. 2014). Alkalinity is used to examine the stability of the digester, as the steady system has an alkalinity between 1000-5000 mg CaCO_3/L (Rahmani et al., 2022). The alkalinity of all the assays ranged between 2400-3600 mg CaCO_3/L , showing the digester's good buffering capacity (Figure 3b). The alkalinity is increased in anaerobic digesters treating protein-rich substrate (sludge) owing to the formation of ammonia which further reacts with CO_2 and forms ammonium bicarbonate. The carbonate ions and CO_2 also contribute to alkalinity. Volatile solids (VS) reduction positively correlated with biomethane production. The maximum VS removal of 54% was observed for the 160°C - 30min THP assay, i.e., 2.6 times higher over control (20.7% VS reduction).



(a)



(b)



(c)

Fig. 3. (a) pH, (b) alkalinity-VFA, and (c) NH_4^+ -N variations in BMP assays

3.3. Fate of organic micropollutants

The effect of the THP process combined with AD on the removal of organic micropollutants from sludge was investigated (Table 1, Figure 4). Among the sixteen THP conditions, the best conditions, i.e., 120°C- 90min, 140°C-60min, 160°C- 30min, 180°C-30min, were selected based on the higher sludge solubilization and methane yield and only these samples were analyzed for organic micropollutants. Along with this, the control (untreated) sludge which was subjected to anaerobic digestion was also analyzed for organic micropollutants before and after the batch studies.

In control sludge, oestrogens concentration was found to be 2270 $\mu\text{g/kg}$ (estrone), 1211 $\mu\text{g/kg}$ (17β -estradiol), and 351 $\mu\text{g/kg}$ (17α -ethinylestradiol), and corresponding removal of 31, 17 and 14% was observed after anaerobic digestion of control. When the sludge was pretreated at

180°C-30min and then subjected to anaerobic digestion, the highest removal (70%) was observed for 17 β -estradiol, followed by estrone (55%) and 17 α -ethinylestradiol (34%) removal. Hamid and Eskicioglu (2013) also reported a notable increase in the removal of estrone and 17 β - estradiol during anaerobic digestion of thermally pretreated sludges (80-160°C) over control. During pretreatment, the polymeric substances in the sludge samples are perhaps liberated into the aqueous phase and become almost biodegradable based on the alterations in their molecular structure under variable pretreatment regimes (Hamid and Eskiciogolu., 2013). Progesterone concentration in the control sludge was 220 μ g/kg, and anaerobic digestion of the control sludge resulted in a 6% removal of progesterone. The THP+AD led to a progesterone removal efficiency of 34%, which is significantly higher than the control. The results indicate that THP pretreatment increased the removal of hormones during anaerobic digestion. Under all redox conditions, degradation of natural oestrogens takes place. Reduction of E1 (estrone) to E2 (17 β -estradiol) and further removal of E2 have been proven under anaerobic conditions. However, an esteric obstruction in EE2 (17 α -ethinylestradiol) does not favor the production of a ketone (as in the case of E2) owing to the occurrence of the ethinyl group in position 17. Hence, its removal ability is lesser over E2. Ruas et al. (2022) reported almost negligible removal of hormones under anaerobic conditions.

Microbial population, bioavailability, and co-metabolic phenomena are some factors affecting the biodegradation of micropollutants (Carballa et al., 2006). During biodegradation, the uptake of micropollutants by the cell takes place in which the structure of the compound plays a crucial role in deciding the resistance of a particular compound to degradation (Luo et al., 2014). Easily degradable compounds include unsaturated aliphatic compounds, compounds with electron-donating functional groups, linear compounds with short-chain acids, and the recalcitrant

micropollutants include compounds with branched chains, compounds with halogen or electron-withdrawing functional groups, and polycyclic compounds (Tadkaew et al., 2011).

For the three antibiotics analyzed in this study, enrofloxacin had the highest concentration (7549 $\mu\text{g/kg}$) in comparison with the other antibiotics, followed by ciprofloxacin (5278 $\mu\text{g/kg}$) and trimethoprim (3237 $\mu\text{g/kg}$) in the control sludge. After THP, the concentration of TMP was below the detection limit (10 $\mu\text{g/kg}$). The results concomitated those reported (75-99%) in earlier studies (Yang et al., 2017; Gonzalez-Gil et al., 2016; Alvarino et al., 2014). The combination of THP and anaerobic digestion resulted in the concentration of TMP being below the detection limit (10 $\mu\text{g/kg}$). Enrofloxacin showed a high removal of 82% after anaerobic digestion of control sludge. The enrofloxacin removal was improved to 99% during anaerobic digestion of THP pretreated sludge at 180°C. The concentration of ciprofloxacin (CIP) in the control sludge was 5278 $\mu\text{g/kg}$, and a 38% removal in CIP was observed after anaerobic digestion. The removal of CIP increased to 85% in anaerobic digestion of 180°C- 30min pretreated sludge. CIP has a low Henry constant of 5.09×10^{-19} ($\text{atm} \cdot \text{m}^3/\text{mol}$). It has carboxylic and piperazinyl groups in the structure, which denotes the stability of the compound. It is not easily volatilized. Hence, sorption and biodegradation could be the possible mechanisms of ciprofloxacin removal under anaerobic conditions. Bioaccumulation of solute into lipids in the microbial cell could result in the sorption of CIP into sludge. Hydrophobicity or electrostatic interactions between the amino acids present in the compound and cell surface play a vital role in sorption. CIP, a hydrophilic compound, has a low K_{ow} ; hence, adsorption through electrostatic attraction could be the possible mechanism behind removal (Ternes et al., 2004).

Carbamazepine is mainly used in the treatment of epilepsy and is also used as a mood stabilizer in the treatment of bipolar disorders (Van Den Brandhof and Montforts, 2010).

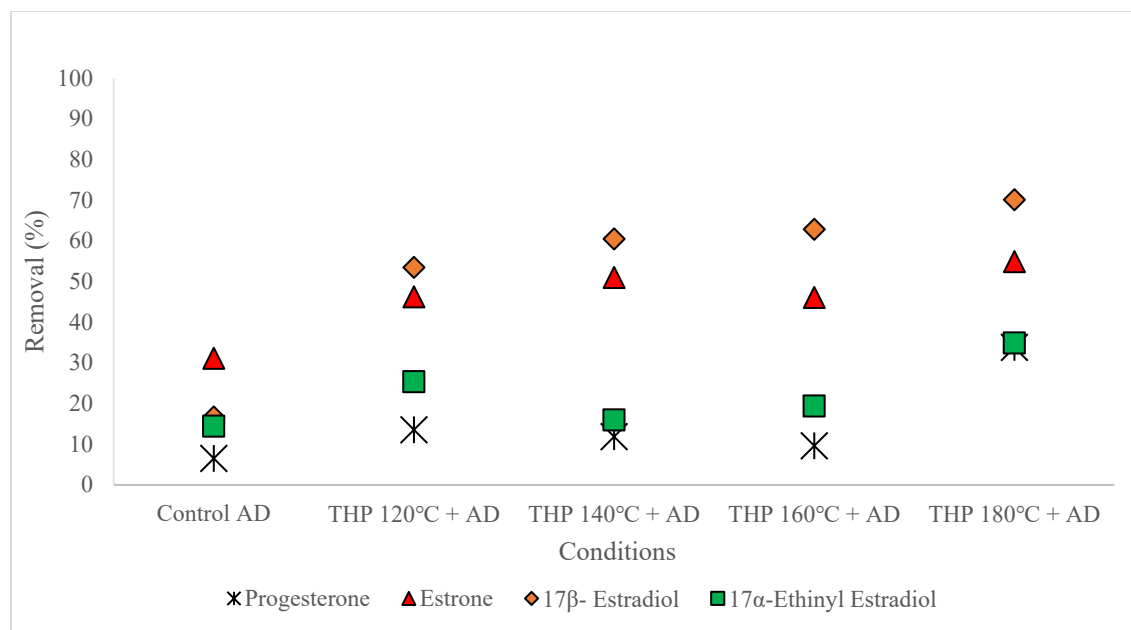
Carbamazepine showed a concentration of 118 µg/kg in control sludge and it was reduced to 78 µg/kg (34% removal) after anaerobic digestion (control). The THP-AD achieved 43% removal of carbamazepine for the sludge pretreated at 180°C for 30min, i.e., 27% higher than the control assay. Earlier studies reported lower removal of carbamazepine (<15%) and reported its resistance to anaerobic digestion (Gonzalez-Gil et al., 2016; Stamatelatou et al., 2003; Alvarino et al., 2014). The recalcitrant nature of carbamazepine to anaerobic digestion could be because of the presence of a pyridine group in its structure. Diclofenac was present in the control sludge at a concentration of 898µg/kg. Only 17% removal of diclofenac was observed during anaerobic digestion. Alvarino et al. (2014) also reported the low removal of diclofenac (<15%) under anaerobic conditions. Carballa et al. (2006) stated that biomass acclimatization favors the biodegradation of diclofenac under anaerobic conditions. For all the THP pretreated conditions, the diclofenac removal was achieved by approximately 65%. The concentration of bezafibrate in the control sludge was observed to be 1626 µg/kg. It was reduced to 796 µg/kg after anaerobic digestion of control sludge. The concentration of bezafibrate was drastically reduced to 94µg/kg after anaerobic digestion of THP sludge pretreated at 180°C for 30min. Overall, THP pretreatment followed by anaerobic digestion improved the degradation of most organic micropollutants studied.

490 **Table 1.** The concentration of micropollutants in raw sludge, anaerobically digested sludge, and THP+ anaerobically digested sludge

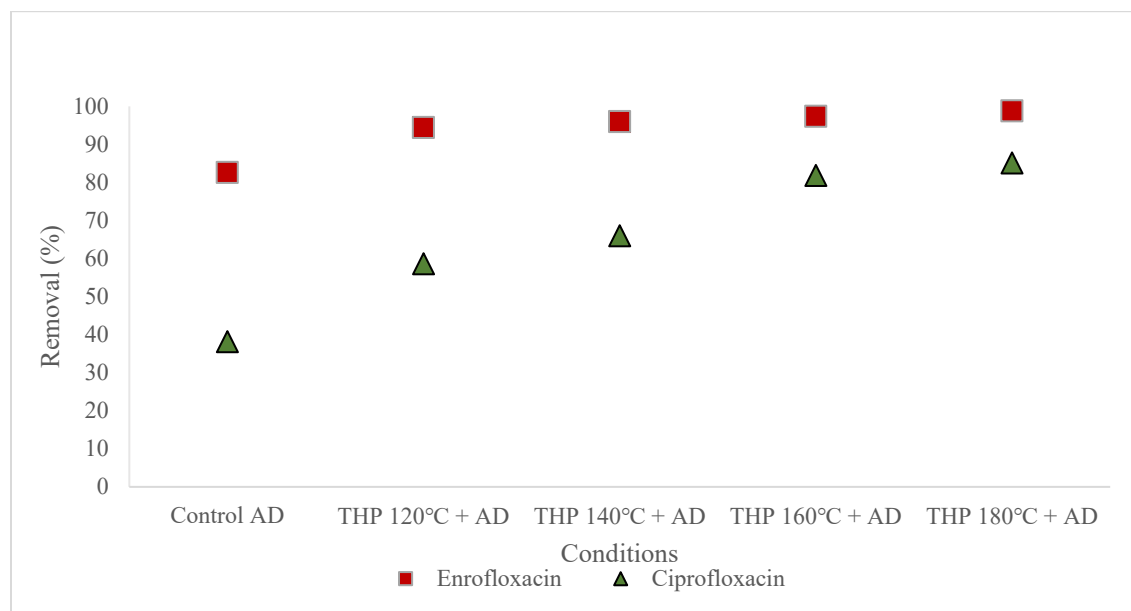
OMPs	Raw Sludge	After AD (Control)	AD of THP Sludge (120°C-90 min)	AD of THP Sludge (140°C-60min)	AD of THP Sludge (160°C-30min)	AD of THP Sludge (180°C-30 min)
Estrone (E1)	2271	1564	1220	1113	1224	1024
17 β -estradiol (E2)	1211	1009	564	479	450	362
17 α -ethinylestradiol (EE2)	352	301	263	296	284	229
Progesterone	221	206	191	194	199	146
Trimethoprim	3237	46	<D.L	<D.L	<D.L	<D.L
Enrofloxacin	7549	1310	420	302	198	91
Ciprofloxacin	5278	3264	2181	1793	958	785
Carbamazepine	118	78	74	70	80	68
Diclofenac	898	748	325	306	326	295
Bezafibrate	1626	796	216	166	119	94

491 *All concentrations are in $\mu\text{g/kg}$, <D.L- Below Detection Limits

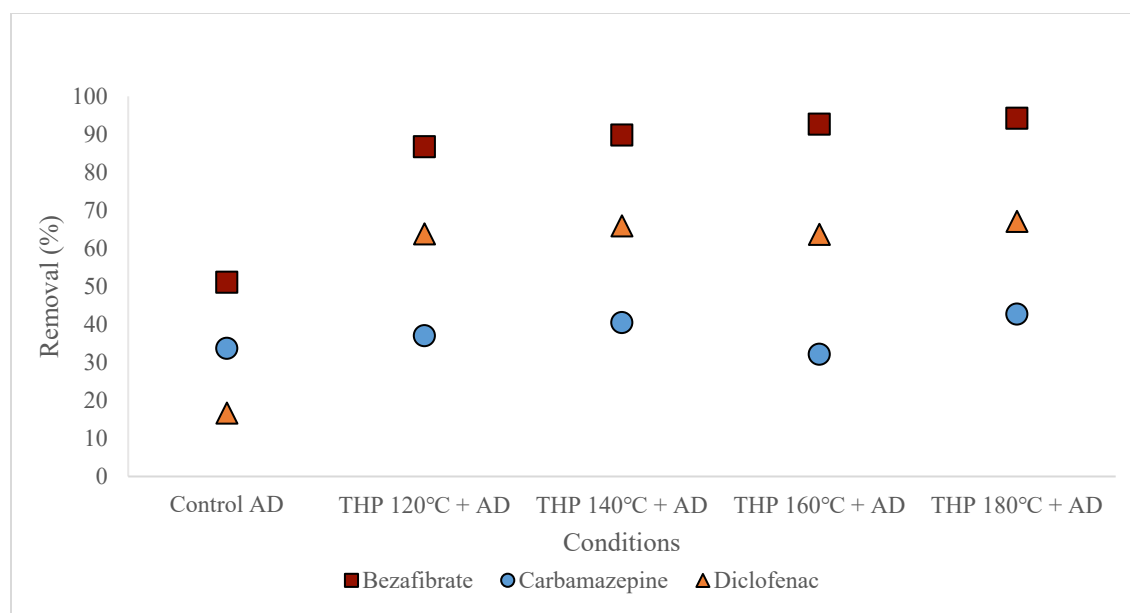
492



(a)



(b)



(c)

Fig. 4. Effect of thermal sludge hydrolysis process mediated AD on (a) hormones (b) antibiotics, and (c) other organic micropollutants

3.4. Microbial diversity analysis

It is a well-known fact that there is a correlation between taxonomic hierarchy and gene functionality of anaerobic microbial communities responsible for biogas synthesis. It can be influenced by feedstock type, temperature, loading rate, ammonia concentration, and other parameters (De Vrieze et al., 2015; Regueiro et al., 2015). Microbial diversity analysis helps identify the community structure responsible for organic mass degradation and methane production. Within the bacterial domain, the most abundant phyla were *Proteobacteria*, *Firmicutes*, *Chloroflexi*, and *Bacteroidetes*, collectively accounting for > 70-80% of bacterial reads (Figure 5a). These phyla have been reported to dominate in anaerobic digesters treating sludge (Riviere et al., 2009). The relative proportion of these dominating phyla varied among control and THP pretreated-anaerobically digested sludge samples from 10-40%. The presence of these bacteria represents the dominance of biomass hydrolyzing

communities. *Proteobacteria* are bacterial phylum (kingdom-eubacteria) that includes purple bacteria involved in the initial biomass degradation, mainly propionate, butyrate, and acetate (Okabe et al., 2007). *Bacteroidetes* are protein-degrading bacteria, acting upon amino acids to produce acetate for acetoclastic methanogens (Okabe et al., 2007). *Firmicutes* are syntrophic bacteria and act upon volatile fatty acids producing H₂ utilized by the hydrogenotrophic methanogens (Deppenmeier et al., 2002). *Chloroflexi* is a green non-sulfur (GNS) bacteria responsible for degrading carbohydrates (Sekiguchi et al., 2001; Blackall et al., 2002).

Operation taxonomic unit (OTU) refers to the primary taxonomic unit referring to a class/genus/species that aids in classifying closely related groups. The most abundant genera were *Pelolinea*, *Brevefilum*, *Arcobacter*, *Pseudomonas*, *Clostridioides*, *Acinetobacter*, *Clostridium*, and *Flavobacterium* (Figure 5b). *Pelolinea* has been reported to dominate during mesophilic AD, controls saccharides fermentation into VFA and H₂, and biodegrades inhibitory phenolic compounds (Rosenkranz et al., 2013). Accumulation of *Clostridioides* (phylum Firmicutes) aids in the enhanced decomposition of major biomolecules such as polysaccharides, proteins, and fats (Martín-González et al., 2011). *Arcobacter* belongs to phylum Proteobacteria, which includes diverse species which are obligate and facultative chemolithoautotrophs as well as heterotrophs. Callbeck et al. (2019) reported that *Arcobacter* sp. dominated in digesters having high sulfide concentration and organic load and can utilize both H₂ and CO₂.

A principal coordinate analysis (PCA) was done to explore dissimilarities between the control and pretreated sludge microbial communities (Figure 5c). The control (untreated) sample clustered far away from all four pretreated samples indicating a more heterogenous microbiome than the thermally THP pretreated sludge. All the THP pretreated samples- 140°C-60 min, 160°C-30min, and 180°C-30min clustered closely, with the former two having similar PC2 values and

the latter two having similar PC1 values. However, the 120°C-90min pretreated sample clustered slightly away from the other three pretreated samples, suggesting differences in microbial community structure of than 140°C-60min, 160°C-30min, and 180°C-30min pretreated samples (Figure 7), which might be due to the lower temperature range when compared to 140°C-60min, 160°C-30min and 180°C-30min.

The alpha diversity was checked using Simpson, Shannon, and Chao-1 indices, and the values are represented in Table 2. These indices estimated maximum microbial diversity in 120°C-90min pretreated sludge as compared to the other three samples (140°C-60min, 160°C-30 min, 180°C-30min). It might be due to slightly elevated temperature and pressure in latter conditions that a specific group of microorganisms could not survive. However, the control sample showed the lowest alpha diversity value among all.

Table 2. Statistical analysis of alpha diversity of microbial communities

Alpha Diversity Index	Control	120°C- 90min	140°C- 60min	160°C- 30min	180°C- 30min
Simpson	0.7902	0.8922	0.833	0.8551	0.8993
Shannon	2.385	3.138	2.532	2.746	2.908
Chao-1	262.7	196.3	163	153.9	130

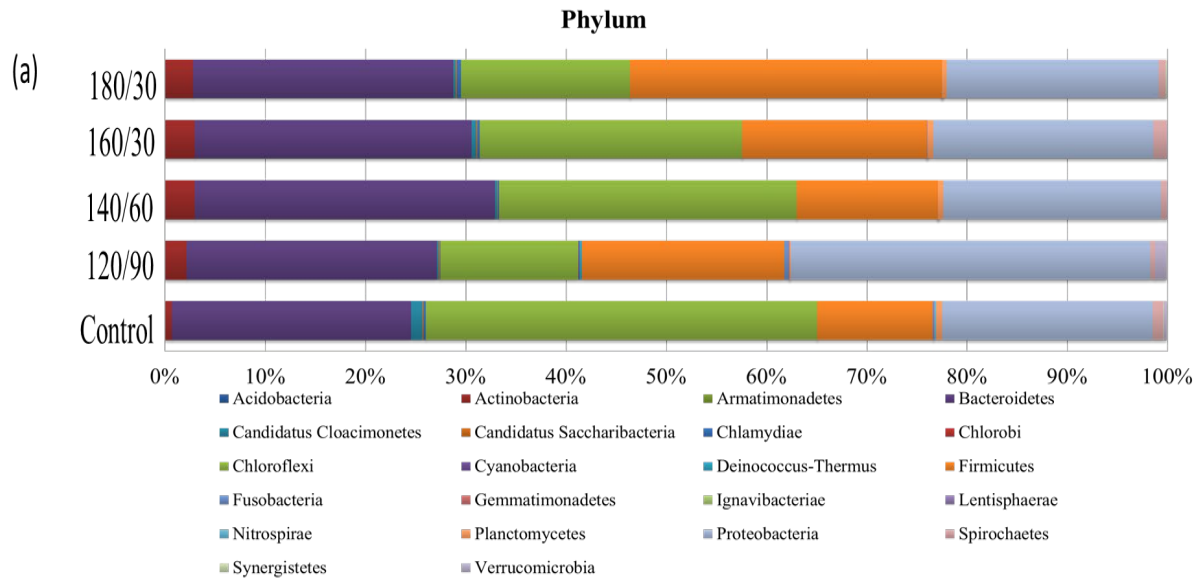
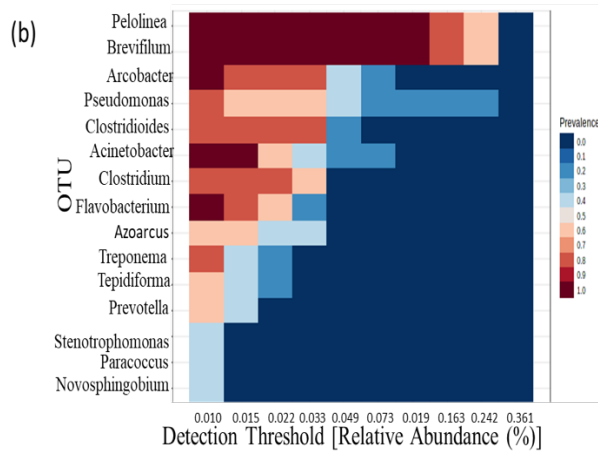
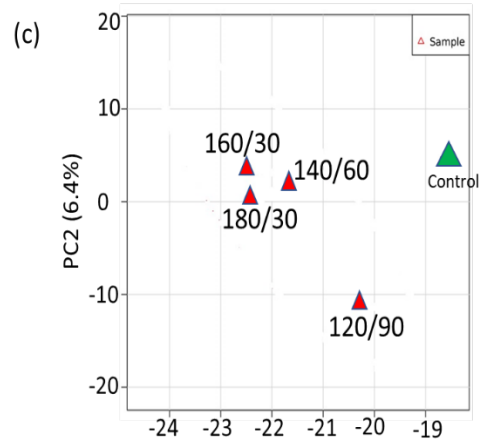


Fig. 5. a) Taxonomic plot analysis at phylum level (x-axis :%abundance, y-axis :sample ID). b) Relative abundance percentages of OTUs at taxonomic genus level, c) Principal coordination analysis (PCA) of microbial communities amongst control and thermally pretreated sludge.

Further, to investigate the effect of different pretreatment conditions on the selective enrichment of bacterial communities, a heat map was generated for comparison at the genus level (Figure 6). The *Proteobacteria*, followed by *Firmicutes*, *Chloroflexi*, and *Bacteroidetes*, were the dominant phylum in pretreated and control sludge assays. Two

methanotrophs, *Methylocystis* and *Methylomonas*, belonging to α -*Proteobacteria* and γ -*Proteobacteria*, respectively, were detected. Among two dominating methanotrophs, *Methylocystis* is a type II methanotroph responsible for carrying methane oxidation in anaerobic digesters. On the contrary, *Methylomonas* is a type II methanotroph, accountable for conducting methane oxidation and might couple it with denitrification. The former dominated in pretreated samples, while the latter showed abundance in control sludge samples. The genera *Pseudomonas*, *Nitrosomonas*, *Arcobacter*, *Geobacter* and *Desulfoglaeba* were among the most abundant *Proteobacteria* and are known to hydrolyze proteins. The genera specifically present in pretreated samples were *Curtobacterium* (Phylum-Acinetobacter) and *Arcobacter*, *Bacillus*, *Comamonas*, *Desulphomicrobium*, *Methylocystis* (Phylum-Proteobacteria). The control assay showed an exclusive abundance of *Nitrospira* (Phylum-Firmicutes) and *Methylomonas* (Phylum-Proteobacteria). The most dominating genera of the phylum *Chloroflexi* included *Brevefilum*, *Pelolinea*, and *Tepidiforma*. They have been reported to ferment a wide range of carbohydrates into fatty acids. *Bacteroidetes* (*Flavobacterium*, *Prevotella*, *Blattabacterium*) were present in both pretreated and control sludge and mainly performed hydrolysis and acidogenesis steps during AD.

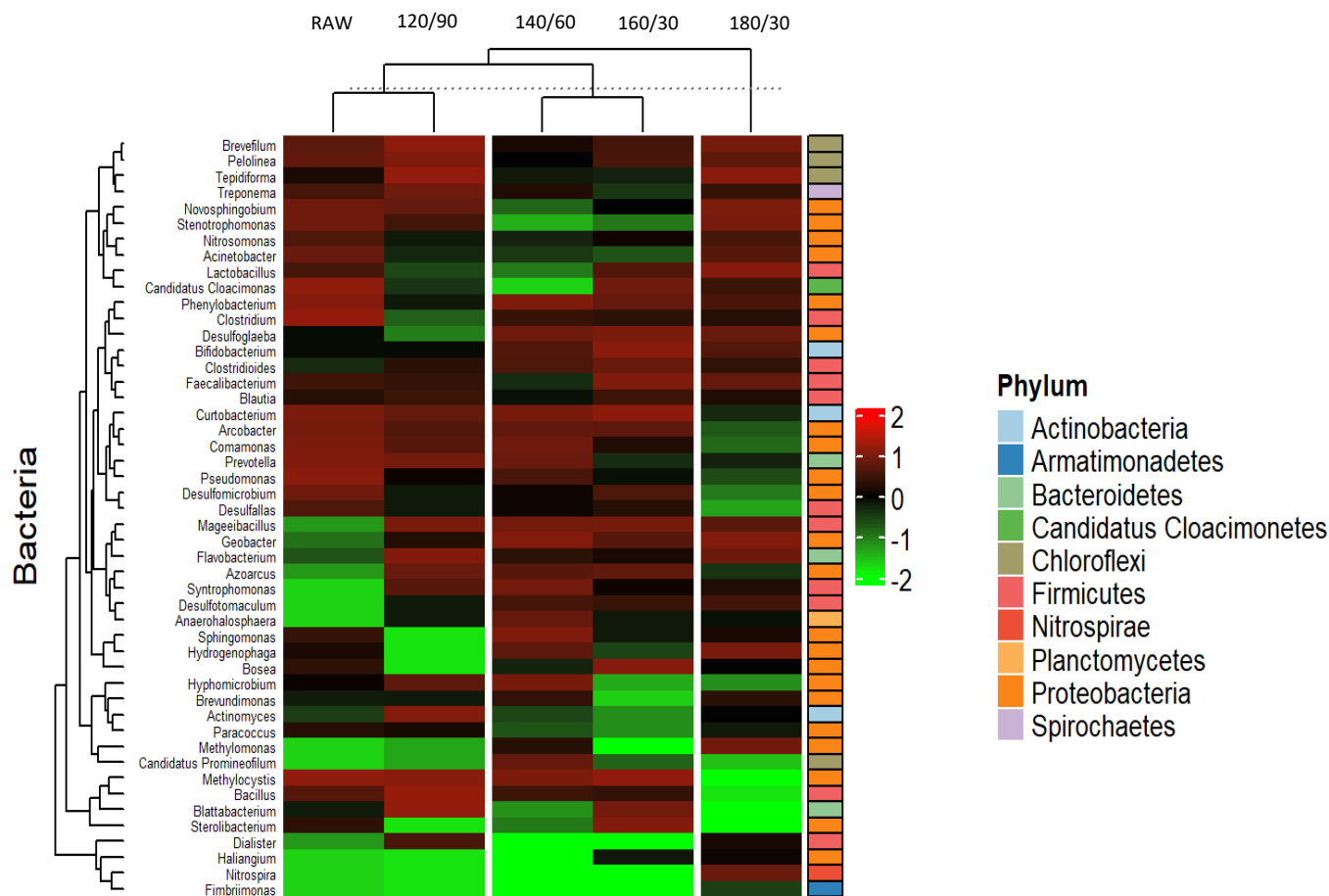


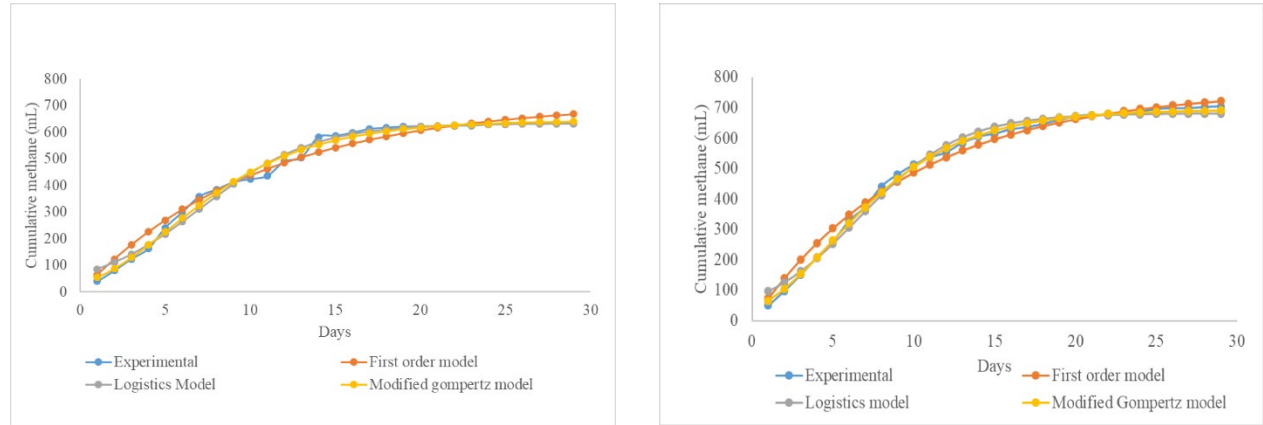
Fig. 6. Heat map of control and THP pretreated sludge assays at the genus level

4. Kinetic modelling

4.1. Model fitting and regression analysis

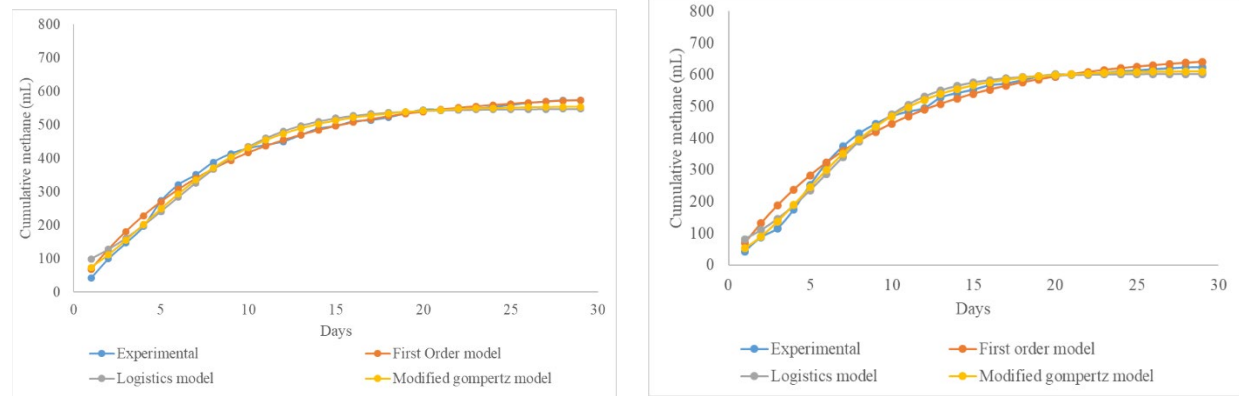
To observe the correctness of the data, the values obtained from the batch assays of THP pretreated sludges were plotted with those obtained with the modeled values for 160°C- 30min, 60min, 90min, 120min. (Figure 7). The R^2 values (0.96-1.0) show that all the kinetic models fit well with the experimental values. The most significant observation during the analysis was a decrease in lag phase time in the THP hydrolyzed samples, which was noted in LM and MGM. A mean of 0-1 days lag phase was observed by LM and MGM in each of the THP pretreated samples, which justifies the fact that the pretreatment

accelerated the hydrolysis stage. Similarly, a high methane production potential was observed in all the THP pretreated sludges, when compared to the control sludge. The trend of methane produced experimentally and by those predicted by the models are also in line (Figure 7).



(a)

(b)



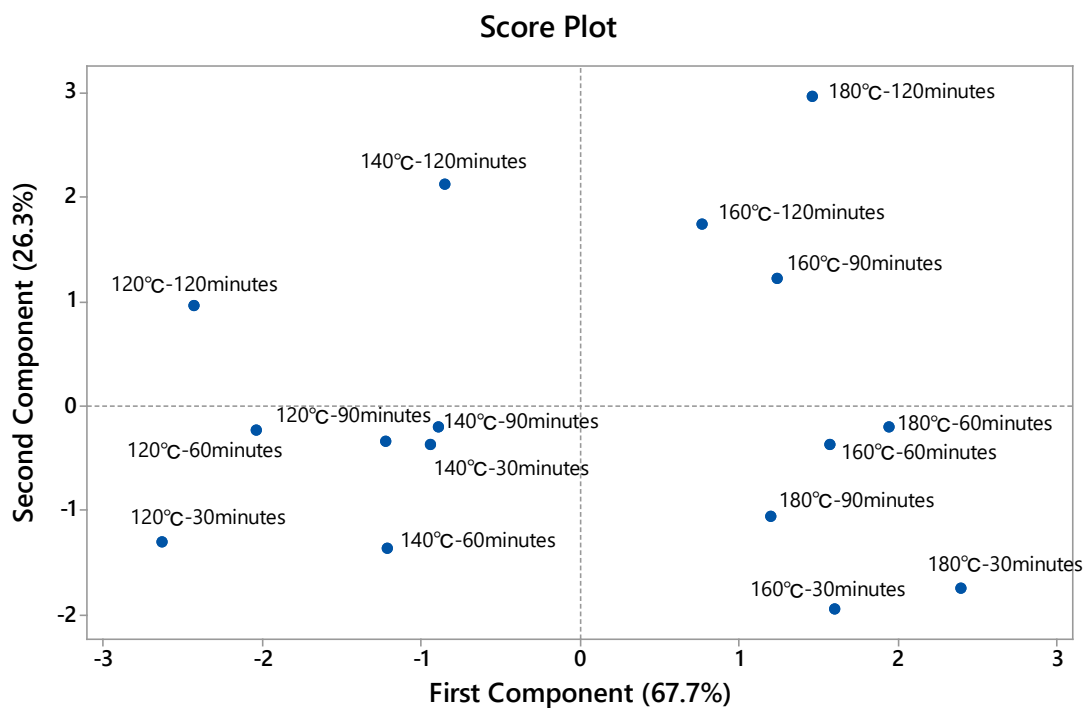
(c)

(d)

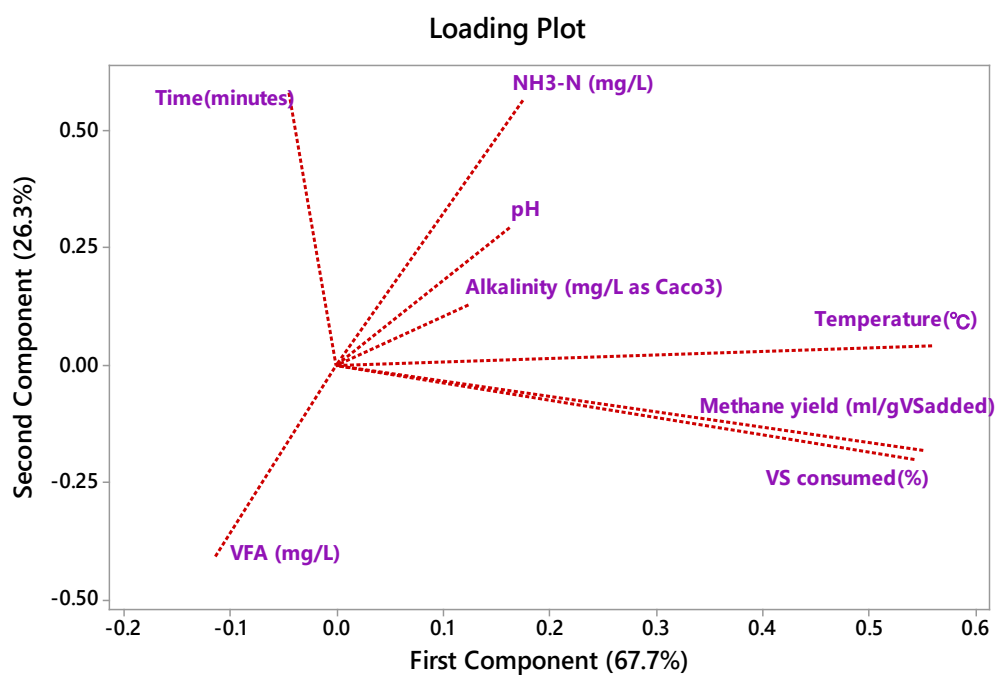
Fig. 7. Time-series plot of experimental and modeled values at 160°C (a) 30min (b) 60min (c) 90min (d)120min

4.2 Principal component analysis (PCA)

611 The principal component analysis (PCA) was used to analyze the difference and likeness among
612 various factors of THP sludges. The PCA loading and score plots have been given in Figure 8
613 (a,b). The score plot indicates the position of the different THP pretreated sludges. The loading
614 plots exhibit the arrangement of several experimental variables in space, established by the first
615 and second principal component analysis dimensionality. The aggregate of the principal
616 components is more than 80%, which implies that the data is significant, and principal component
617 analysis can be performed on this data. The score plots show that the digestate parameters for the
618 thermally hydrolyzed sludge at 120°C and 140°C at the end of 29 days were similarly correlated
619 since they lie in the same quadrant. A similar trend was observed for samples pretreated at 160°C
620 and 180°C. The loading plots indicate that the digestate parameters varied with increasing
621 temperature. As the treatment temperature increased from 120 to 180°C, alkalinity, $\text{NH}_4^+\text{-N}$, and
622 pH also showed an increasing trend, as seen in the first quadrant. However, the VS removal (%)
623 and methane yield increased up to the treatment temperature of 160°C and reduced afterward. It
624 can be justified by observing the loading plot where the methane yield and VS removal are on the
625 vertical quadrant's positive side but the horizontal quadrant's negative side. Although the VFA
626 concentration of the digestate was under limits, it did not follow a trend with an increase in
627 temperature.



(a)



(b)

Fig. 8. (a) Score plot and (b) Loading plot of the thermally-hydrolysed batch assay digestate

5. Conclusions

Among, all the pre-treatment conditions studied, 160°C- 30 min showed the best efficiency in terms of cumulative methane yield (507 mL CH₄/g VS_{added}) with a VS removal of 54% making it the optimum condition for THP pretreatment. Higher pretreatment temperatures (>140°C) and shorter reaction times (30 min) favoured the methane yield over longer reaction times (120 min). THP pretreated sludges showed >80% removal of Trimethoprim, Enrofloxacin, Ciprofloxacin, and Bezafibrate and <50% removal of Carbamazepine, 17 α -ethinylestradiol, and Progesterone. THP followed by anaerobic digestion aided in the biodegradation of the organic micropollutants. *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were the most abundant phylum. The samples pretreated at 120°C-90 min showed the highest microbial diversity (145 OTUs) followed by a successive decrease in diversity in the order 140°C-60 min (140 OTUs), 160°C-30 min (130 OTUs) and 180°C-30 min (97 OTUs). Thus, thermal hydrolysis significantly improved the methane yield and removal of micropollutants during anaerobic digestion. The pathways involved in organic micropollutant degradation need to be investigated in future studies.

Credit authorship contribution statement

Gowtham Balasundaram: Conceptualization, Visualization, Methodology, Writing – original draft, Data curation. Pallavi Gahlot: Conceptualization, Visualization, Methodology, Writing – original draft, Data curation. Banafsha Ahmed: Data curation, Writing – review & editing. Pinakshi Biswas: Methodology, Data curation, Writing. Vinay Kumar Tyagi: Conceptualization, Visualization, Resources, Methodology, Writing – review & editing, Critical suggestions,

Supervision. Vinod Kumar: review & editing, critical suggestions. A.A. Kazmi: Writing – review & editing, critical suggestions, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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