Effects of mild thermal pre-treatment combined with Hydrogen peroxide and Iron addition on Waste Activated Sludge

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Effects of mild thermal pre-treatment combined with Hydrogen peroxide and Iron addition on hydrogen production from Waste Activated Sludge

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By

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Cover page : Picture of Themista® at Kralingseveer

उद्यमेन हि सिध्यन्ति कार्याणि न मनोरथैः।
न हि सुप्तस्य सिंहस्य प्रविशंति मुखे मृगाः॥

("Success is achieved through hard work, not merely by wishful thinking. Just as a deer does not wander into the mouth of a sleeping lion, accomplishments do not come to those who are idle")

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Abstract

Sewage sludge production poses a significant global challenge, with anaerobic digestion (AD) being the most widely employed method to manage this issue. However, since hydrolysis is the rate-limiting step in AD, pretreatment of sludge can aid in enhancing the process.

The current study aimed to evaluate sludge solubilization and gas production—particularly bio-hydrogen (bio-H_2) production—following low-temperature thermal pretreatment (LTTP) at 55°C. This thesis replicates the full-scale setup of the Themista® system, which employs a two-stage thermal pretreatment process with 55°C and 70°C as the heating stages, operating in a semi-continuous mode. Four distinct pretreatment experiments were conducted to assess sludge solubilization and gas production: LTTP , LTTP with H_2O_2 at 55°C, LTTP with $FeCl_2$ at 55°C, and LTTP with both H_2O_2 and $FeCl_2$ at 55°C.

The results demonstrated that both thermo-chemical and thermal pretreatments exhibited similar soluble chemical oxygen demand(sCOD) release patterns. A shift from tightly bound to more soluble fractions of extracellular polymeric substances (EPS) was observed post-pretreatment. Gas production was noted only at 55°C across all conditions, with bio-H_2 being produced under each pretreatment. Notably, thermal pretreatment with $FeCl_2$ resulted in an average H_2 COD/tCOD ratio that was 24 times higher than that of thermal pretreatment alone. However, the average sludge to gas COD percentage for all pretreatments remained below 0.06%, with the highest of 0.1% observed in the thermal treatment with $FeCl_2$. Additionally, significant increases in BMP and a sludge COD to CH_4 conversion percentage of 75% were observed post-treatment with thermal pretreatment combined with $FeCl_2$.

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1.Introduction

Sewage sludge, a significant byproduct of wastewater treatment processes, presents substantial challenges for environmental management (El-kebeer et al., 2024). Globally, municipal wastewater treatment plants generate approximately 45 million dry tons of sewage sludge annually, with the European Union, the United States, and China contributing to 40-70% of this total (Giwa et al., 2023; Guo et al., 2015). As populations grow and WWTP regulations become stricter, the volume of sludge is expected to increase. Given its high organic content and the presence of various contaminants, the proper management and disposal of sewage sludge are critical to mitigating environmental pollution, necessitating the pursuit of sustainable and viable solutions.

Anaerobic digestion (AD) is widely employed for managing sewage sludge due to its suitability as a substrate for the process, largely owing to its high organic content (Wainaina et al., 2020). AD operates through four sequential stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, culminating in the production of biogas primarily composed of CH_4, CO_2, H_2S, and H_2 (El-kebeer et al., 2024). However, the hydrolysis stage often serves as the rate-limiting step, resulting in extended residence times and increased costs in wastewater treatment operations (El-kebeer et al., 2024).

Enhancing the hydrolysis process through pretreatment is a well-established strategy to overcome this bottleneck. Pretreatment disrupts cell structures, facilitating the release of organic substances (Bougrier et al., 2005), thereby accelerating hydrolysis, reducing retention times, and lowering the overall costs associated with wastewater treatment. Various pretreatment technologies are available, categorized into physical, chemical, and biological methods (El-kebeer et al., 2024). Among these, thermal pretreatment (TP) has garnered considerable attention and has been implemented at laboratory, pilot, and industrial scales (Mirsoleimani Azizi et al., 2024). TP is classified based on operating temperatures into low-temperature TP (LTTP; <100°C) and high-temperature TP (HTTP; ≥100°C) (Kor-Bicakci & Eskicioglu, 2019; Pilli et al., 2015). While HTTP is more effective in solubilizing sludge, it can lead to the release of recalcitrant and inhibitory substances, such as melanoidins (Mohammad et al., 2024). In contrast, LTTP offers higher net energy advantages due to its lower energy input requirements (Pilli et al., 2015).

AD also presents the potential for producing valuable bioproducts such as biohydrogen (bio-H_2). Generating bio-H_2 from sewage sludge by integrating sludge digestion with H_2 production not only addresses sludge management challenges but also promotes a cleaner alternative for hydrogen production (Yao et al., 2018). Bio-H_2 is a highly valuable energy source, known for its high energy density and clean fuel properties (Edwards et al., 2008). Bio-H_2 can be produced through photo fermentation by photosynthetic bacteria or dark fermentation by various groups of heterotrophic bacteria (Kothari et al., 2012; Shimizu et al., 2019). Dark fermentation, which involves obligate and facultative anaerobes producing H_2 in the absence of light and oxygen (Kamran, Muhammad, 2021).It

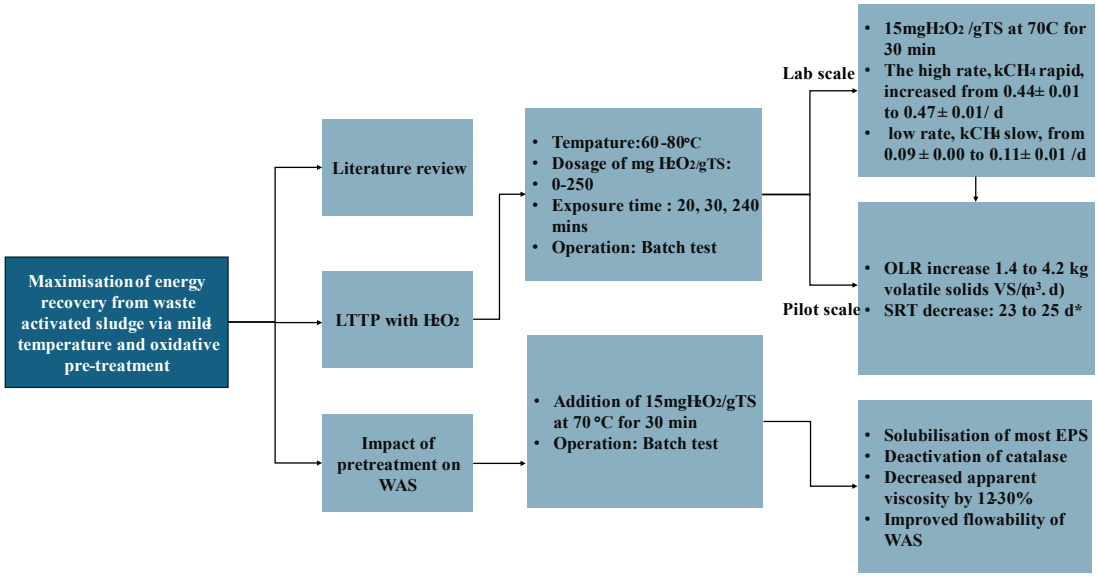
is considered more economically viable and promising compared to photo fermentation (Argun & Dao, 2017; Cao et al., 2022a). Since H_2-rich biogas can be generated during the acidogenic stage of AD through the conversion of volatile fatty acids (VFAs), producing hydrogen via dark fermentation within the AD pathway represents a promising alternative for energy generation from sewage sludge (Albini et al., 2019; El-Khateeb et al., 2023).

Another perspective worth considering is the potential for gas production, particularly bio-H_2 production, during the pretreatment stage of sewage sludge processing. This potential is especially significant when pretreatment is conducted at thermophilic conditions within high-loaded systems operating at short solids retention times (SRTs). However, there is limited literature exploring this aspect of thermophilic pretreatment. This thesis seeks to explore the possibility of gas production, especially H_2 production, at the pretreatment stage. The study evaluates the impact of semi-continuous, two-phased, thermal and thermo-chemical pretreatment on sludge hydrolysis and gas production, with reactors operating at 55°C and 70°C and incorporating the addition of H_2O_2 and/or $FeCl_2$.

1.1 Background

The background for this thesis was established by the doctoral thesis of Adrian Gonzalez from the Delft University of Technology (TU Delft) and the Themista® technology developed by Royal HaskoningDHV (RHDHV). In this section, a summary of Adrian's doctoral thesis (relevant to this study) given by Figure 1 and results from the full-scale project operated by RHDHV are provided for the reader's reference.

Adrian Gonzalez,(Gonzalez, 2022a) extensively investigated the effects of LTTP and further combined with hydrogen peroxide(H_2O_2) dosing on sludge hydrolysis. Figure 1 summarises the work done by Adrian Gonzalez and the key findings obtained.



*pilot scale was a two stage compartmentalised anaerobic digestor where sludge pretreated at 70°C with 15mg/gTS H_2O_2 was added, effect can be attributed to the pretreatment and the design of the AD reactor.

Figure 1 The summary of Gonzalez, (2022a) with key findings after each experiment

The key findings obtained which were that the optimum dose to improve hydrolysis and methanation was 15mg/gTS of H_2O_2 dosed at 70 °C. It is to be noted that the H_2O_2 used is 30% w/w(Gonzalez et al., 2022).The Themista® technology was developed jointly by RHDHV and TU Delft. Figure 2 provides a schematic representation of the first full scale establishment of Themista® at Kralingseveer Rotterdam, Netherlands operated by RHDHV.

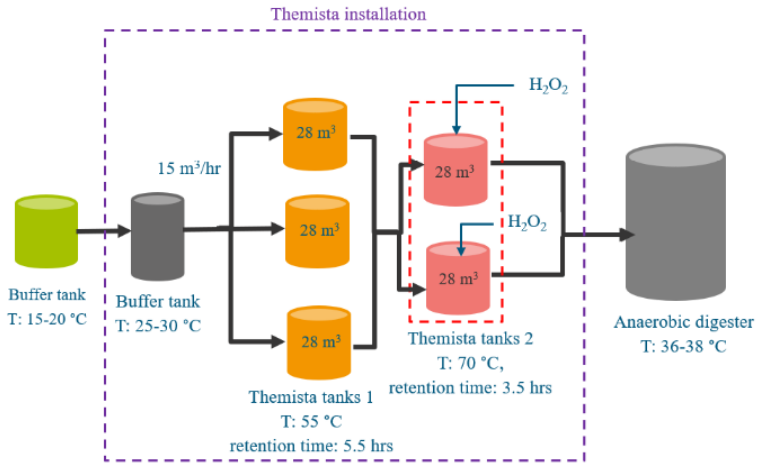


Figure 2 The full-scale establishment of Themista® at Kralingseveer

In full-scale implementation, the Themista® design is semi-continuous thermo-chemical LTTP, with the heating step divided into two stages, initially reaching 55°C before progressing to 70°C. It is positioned after the sludge thickener and before the anaerobic digestor. The thickened sludge enters the buffer tank, then it is pumped to the first heating step at 55°C which has a residence time of 5.5hrs, then the sludge is pumped to the second heating step at 70°C which has a residence time of 3.5hrs. The 30% w/w H_2O_2 of 15mg/gTS is dosed at 70°C.

However, operational issues surfaced as excessive H_2 production at 55 °C tank was observed which led to the shutdown of the full-scale plant. Further investigation revealed that dark fermentation was occurring within the reactors. This prompted the collaboration with TU Delft to explore the potential for harvesting bio-H_2 at 55 °C for later utilization in enhancing bio-methanation.

1.2 Research scope of the current study

Based on the findings from RHDHV, the research scope of this master's thesis was identified as described in the following paragraphs.

The semi-continuous Themista® configuration was replicated in the lab scale. The primal focus was to understand how sludge hydrolysis during AD and H_2 production from dark fermentation during AD can be improved using LTTP or LTTP combined with other technologies. The four LTTP configurations that were explored in this thesis were: LTTP, LTTP combined with dosing H_2O_2 at 55°C, LTTP combined with dosing $FeCl_2$ at 55°C and finally, LTTP combined with dosing of $FeCl_2$ and H_2O_2 at 55°C. Addition

of Fe(II)(here in form of $FeCl_2$) can enhance the enzymes which are responsible for H_2 production from dark fermentation(Cai et al., 2018; Elreedy et al., 2019; Kamran, Muhammad, 2021; X. Zhao et al., 2017a). Furthermore, the combination of Fe (II) and H_2O_2 can lead to Fenton like reactions(Jerez et al., 2022; Pilli et al., 2015a; Zhou et al., 2015). Therefore, to study the above, the combination of LTTP and $FeCl_2$ and LTTP with $FeCl_2$ and H_2O_2 were chosen.

The above research scope is supported by the following research questions:

1. How does a two-phased thermal pretreatment at 55°C impact the efficiency of sludge hydrolysis and the quality and quantity of gas production?
2. How does a two-phased thermal pretreatment at 55°C with the addition of $FeCl_2$ and H_2O_2impact the efficiency of sludge hydrolysis and the quality and quantity of gas production?

2.Theoretical background and research gap

2.1 Anaerobic digestion

AD is a biological process that converts complex organic matter into CH_4 and CO_2 through four primary stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The process begins with hydrolysis, where organic insoluble compounds are broken down into soluble organic compounds (Obileke et al., 2020). A critical factor in facilitating hydrolysis is the production of hydrolytic enzymes. Although these enzymes are produced by microorganisms, their quantity and conversion rate are often insufficient to fully decompose complex organic materials, rendering hydrolysis the rate-limiting step in anaerobic digestion (Atelge et al., 2020).

Following hydrolysis, the process enters the acidogenesis stage, where the soluble compounds generated are further converted into VFAs, alcohols, H_2, CO_2, and other by-products, such as lactic acids, by both hydrolytic and non-hydrolytic microorganisms. Acidogenesis is recognized as the most rapid conversion step in the anaerobic digestion process(van Lier et al., 2020). The specific end products of acidogenesis depend largely on the conditions within the reactor. When scavenging organisms such as methanogens effectively remove H_2, acetate becomes the predominant end product. However, if methanogenesis is inhibited and H_2 accumulates, more reduced compounds such as propionate and butyrate are likely to form (van Lier et al., 2020).

The third stage, acetogenesis, involves the anaerobic conversion of VFAs and alcohols produced during acidogenesis into acetate, H_2, and CO_2 by acetogenic bacteria. Propionate and butyrate are the most common substrates utilized in acetogenesis, serving as key intermediates in the anaerobic digestion process (van Lier et al., 2020). Research on acetogenic conversions has highlighted the essential, narrow associations required between H_2-producing acetogenic bacteria and H_2-consuming methanogenic bacteria, which regulate H_2 levels within the digester environment (van Lier et al., 2020). This regulation is crucial, as the fermentative reactions become thermodynamically unfavourable at elevated H_2 concentrations. However, under stabilized digestion conditions, the hydrogen partial pressure is maintained at an extremely low level, typically below 10^{-4} atm, due to the effective uptake of hydrogen by methanogens or sulphate-reducing bacteria (SRB) (van Lier et al., 2020). Methanogens exhibit a high affinity for molecular hydrogen, ensuring the continuation of the hydrogen-producing acetogenic reactions.

Methanogenesis is the final step in anaerobic digestion, where acetate, H_2, and CO_2 are converted to gaseous CH_4 and CO_2. This step is accomplished by two groups of bacteria: acetotrophic methanogens and hydrogenotrophic methanogens. Acetotrophic methanogens decarboxylate acetate to form CH_4, while hydrogenotrophic methanogens reduce CO_2 using H_2 as an electron donor (van Lier et al., 2020).The complete overview of the AD process is given by Figure 3

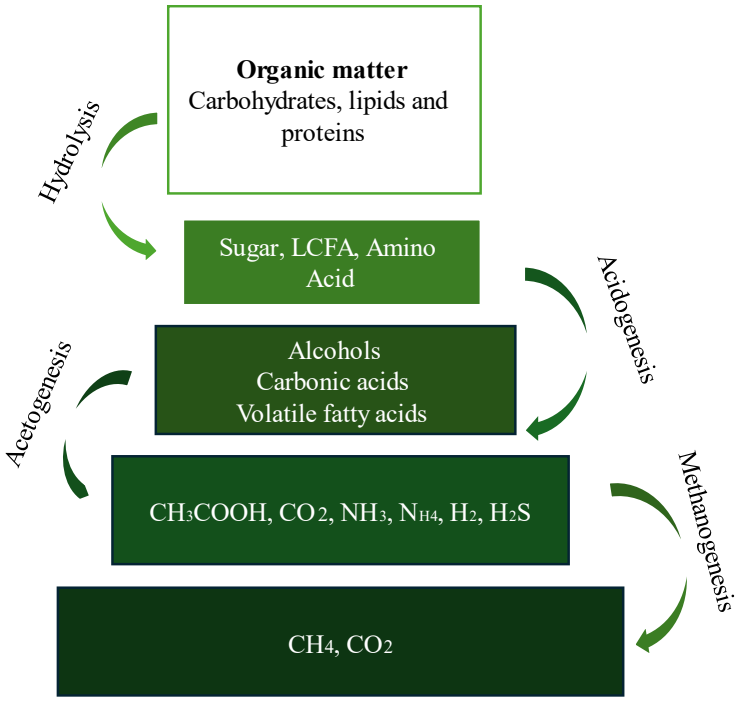


Figure 3 The various steps involved in AD adapted from (Atelge et al., 2020; Obileke et al., 2020)

2.2 Pretreatment

The limited availability of soluble organics and the slow hydrolysis step in AD often lead to large reactor sizes and result in low CH_4 yields(Atelge et al., 2020). Various treatment methods have been developed to address these challenges, with pretreatment of sludge being one of the most effective. Pretreatment enhances substrate availability by subjecting sludge to physical, chemical, and biological treatments that facilitate the solubilization of complex, insoluble organics (Atelge et al., 2020).The goal of pretreatment is to achieve specific criteria: reducing particle size, increasing porosity, enhancing degradability and solubility, eliminating inhibitory substances, and minimizing energy input to ensure cost-effectiveness (J. Singh et al., 2015).

Pretreatment methods are broadly classified into physical/ mechanical, chemical, thermal and biological (Carrère et al., 2010). Figure 4 gives an overview of the commonly reported pretreatment methods. Each method has distinct advantages and limitations regarding efficacy, energy consumption, and cost-effectiveness.

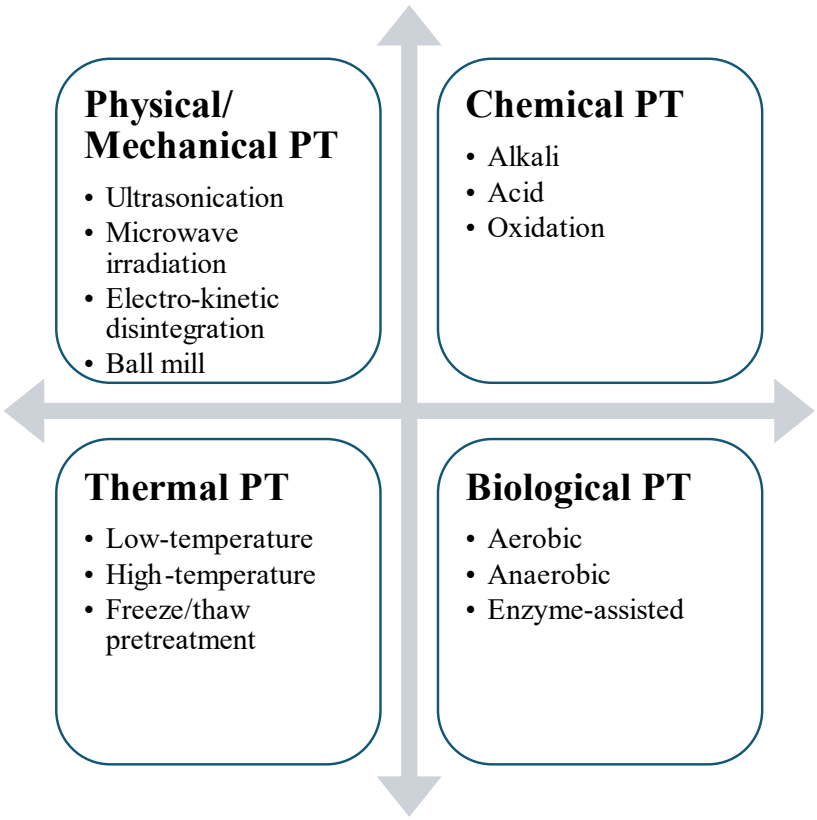


Figure 4 The various pretreatment methods available adapted from (Khanh Nguyen et al., 2021a) PT stands for pretreatment

2.3 Thermal pretreatment

Among the various pretreatment techniques, thermal hydrolysis has garnered significant attention and has been successfully applied at laboratory, pilot, and industrial scales. In the process of thermal pretreatment, heat ruptures the extracellular polymeric substances (EPS) network, which in turn leads to cell lysis. This leads to the release of the organic material(Mohammad et al., 2024). Many WWTPs globally have implemented thermal hydrolysis as a pretreatment in full-scale processes to improve sludge treatment (Bougrier et al., 2005b) This technique is considered to have a high technology readiness level (TRL), often rated at 9, indicating its advanced development and widespread full-scale implementation (Carrere et al., 2016a).

The primary motivation for adopting thermal pretreatment is to enhance the kinetics of AD and increase methane recovery. However, other benefits include improved dewaterability of residuals, pathogen removal for land application of digestate, reduction of scum and foaming during digester operation, decreased odour generation potential of digestate, and reduced viscosity of the digestate (Bougrier et al., 2005b; Carrere et al., 2016). One notable advantage of thermal hydrolysis is the potential for energy recovery. The heat applied during thermal hydrolysis can be captured and reused through heat exchangers for heating additional feedstock, significantly improving energy efficiency and reducing pretreatment costs (Carrere et al., 2016b).

Thermal pretreatment can be categorized based on temperature range and treatment duration. Mild-thermal or low-temperature thermal pretreatment (LTTP) involves applying temperatures between 55°C and 100°C for minutes to several hours. In contrast, high-temperature thermal pretreatment (HTTP), or thermal hydrolysis, uses temperatures exceeding 100°C LTTP, typically conducted at 60-70°C, has shown enhanced hydrolysis efficacy (Gonzalez et al., 2018).

2.3.1 High thermal pretreatment (HTTP)

This pretreatment method involves heating sludge to temperatures above 100°C, which promotes the breakdown and solubilization of organic materials. When sludge is thermally treated at temperatures between 125°C and 175°C, it disrupts cell wall and membrane bonds, making proteins accessible for biodegradation(A. M. Aboulfotoha, E. H. El Goharya, 2015).

Multiple studies have reported the impact of this pretreatment on increasing the sludge solubilisation and biogas production(Barber, 2016; Pilli et al., 2015c). Bougrier et al. (2005a) found that biogas production from the soluble fraction was higher than from the particulate fraction when treated at 95°C to 170°C. The increase in biodegradation was due to the transfer of organic matter from the particulate fraction to the soluble fraction, rather than an increase in the biodegradability of each fraction (Bougrier et al., 2008). Climent et al., (2007) reported that at 175 °C the COD solubilisation increased from 11.25% to 15.1% to 25.1% within 60-240 mins of application. Carrère et al., (2008) pretreatment at 190°C increased biogas production by 150%. HTTP is widely used at full scale; Table 1 gives an overview of all the full-scale HTTP technologies.

Table 1 The full-scale HTTP technologies widely available globally, adapted from (Mohammad et al., 2024)

Technology	Operation temperature	Mode of operation	Process parameters
CambiTHP®/Cambi ASA, Norway	160–180 °C	Batch	6 bar, 20-30 mins
BioThelys™/Veolia, France	130–165 °C	Batch	8-15 bar, 30min
Exelys™/Veolia, France	130–165 °C	Continuous	8-15 bar, 30min
TurboTec®/Sustec, Netherlands	140 °C	Continuous	-
Haarslev/Haarslev Industries, Denmark	165 °C	Continuous	6 bars, 20-30 mins
LysoTherm™/ELIQUO, Netherlands	140–170 °C	Continuous	15-30 mins

HTTP leads to higher solubilisation, higher protein degradation and complete pathogen destruction without chances of reactivation(Khanh Nguyen et al., 2021a). However, it has few disadvantages as high energy requirement, mineralisation of organic compounds or production of Millard reaction products which can affect anaerobic digestion(F. Huang et al., 2021a; Pavez-Jara et al., 2023).

2.3.2 Low Temperature Thermal Pretreatment (LTTP)

LTTP or mild thermal pretreatment typically involves applying temperatures between 55°C and 100°C for a duration ranging from a few minutes to several hours. This method is considered a viable alternative due to its effectiveness in sludge reduction and potential to improve biogas production (Kumar Biswal et al., 2020). This method can stimulate thermophilic bacteria, solubilize organic particles, and improve biodegradability (Khanh Nguyen et al., 2021a).Applying low-temperature pretreatment can also remove pathogens from sludge, De los Cobos-Vasconcelos et al. (2015) investigated producing class A biosolids (solids which have non-detectable pathogen levels) with LTTP at 70°C.

The primary operational parameters are temperature and application time, which result in the disintegration of cell membranes and subsequent solubilization of organic compounds (Nazari et al., 2017). Studies report the impact of LTTP on improving solubilisation and biogas production. (Nazari et al., 2017) reported a soluble Chemical Oxygen Demand(sCOD) increase of 18.3% after the LTTP at 80°C and 5 hrs of application time. Liao et al. (2016a) reported a sCOD disintegration rates of 9.1%, 13% and 16.6% after LTTP at 60,70 80°C respectively.

LTTP is considered to be economic compared to HTTP as it has a lesser energy input and does not need elevated pressure application compared to the HTTP. It can also cause pathogen deactivation. However, the COD and protein solubilisation may not be as high as HTTP. Reactivation of pathogens can happen(Khanh Nguyen et al., 2021a; Mohammad et al., 2024b).

2.4 Chemical pretreatment

Chemical pretreatment can be a promising method for degradation of complex organic material. Addition of chemical reagents such as alkali, acid and oxidants help in improving the cellulose biodegradability, promoting hydrolysis and biogas production(Khanh Nguyen et al., 2021b). Figure 5 is an overview of the commonly reported chemical pretreatment methods used and the principle of application.

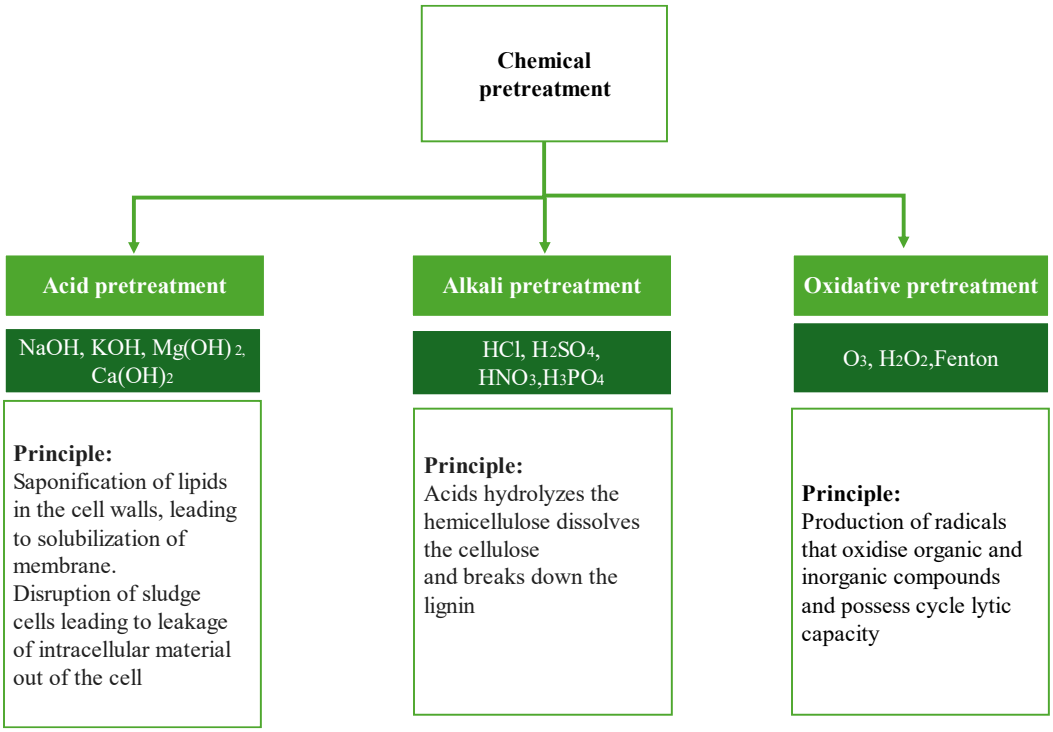


Figure 5 The commonly used chemical pretreatments as reported by (Khanh Nguyen et al., 2021b)

In this study the oxidative pretreatments such as H_2O_2 and $FeCl_2$ with H_2O_2(Fenton) are considered

Mechanism of peroxidation pretreatment

The activation by transition metals, microwave (MW), ultrasound (US), electrolysis, or light irradiation, or heat, H_2O_2 can be converted into highly oxidative hydroxyl radicals (Guan et al., 2018). These strong oxidative hydroxyl radicals are capable of oxidizing sludge flocs and EPS. The destruction of sludge flocs and EPS leads to the release of interstitial water trapped within the flocs and water bound to EPS. Furthermore, the functional groups within sludge EPS are oxidized by hydroxyl radicals, which reduces the extent of π-electron systems, decreases the number of aromatic rings, and alters conjugated bonds within the chain structure, converting linear ring systems into non-linear configurations(Zhen et al., 2012). The collapse of EPS also results in the breakdown of the sludge matrix, thereby facilitating the oxidation of microbial cells The mechanism is given by the following Figure 6.

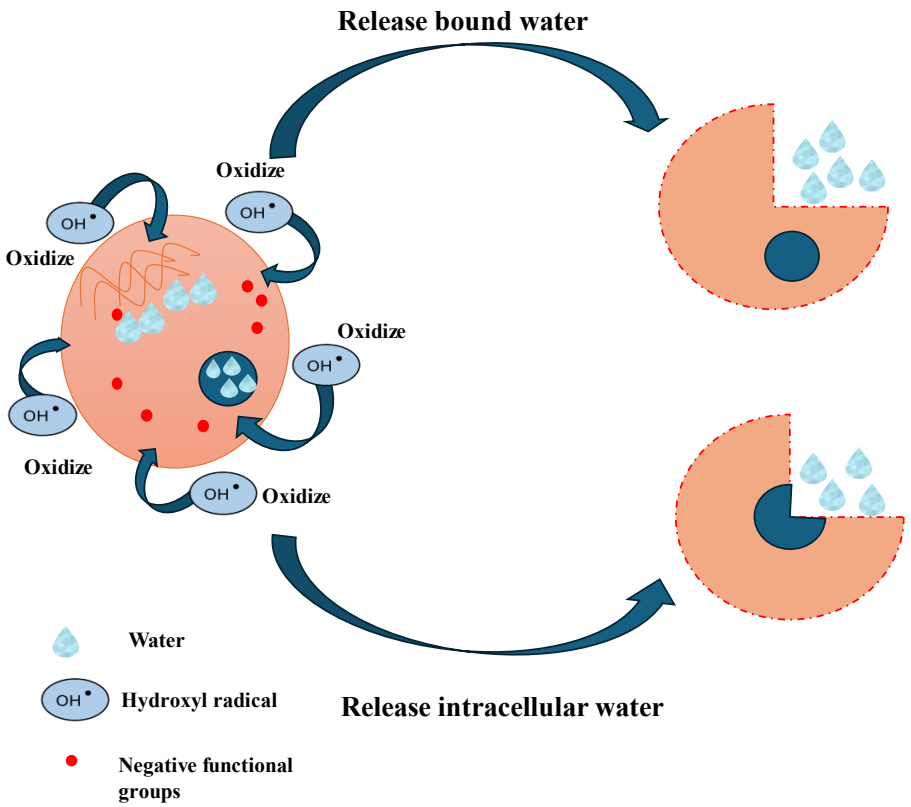


Figure 6 The mechanism of H_2O_2 and Fenton reactions on sludge adapted from(Guan et al., 2018)

A combination of H_2O_2 and ferrous ions, commonly referred to as the Fenton process, is widely utilized in advanced oxidation techniques(Khanh Nguyen et al., 2021). This process involves the reaction of H_2O_2 with Fe^{2+} to generate highly reactive hydroxyl radicals (·OH). Exothermically. These hydroxyl radicals, with a higher oxidation potential of +2.80 V in acidic conditions (pH of 3), surpass the oxidation potentials of H_2O_2 (+1.36 V) and ozone (+2.07 V) (Neyens & Baeyens, 2003; Pilli et al., 2015b). During the reaction, H_2O_2 oxidizes iron catalyst $Fe^{2+}$to Fe^{3+} given by Equation 1. The decomposition of H_2O_2 leads to the generation of hydroxyl radicals and hydroxyl anions (Pham et al., 2011a). The hydroxyl radicals react with Fe^{2+}, resulting in chain termination as described by Equation 2, producing Fe^{3+} and hydroxyl anions. In the presence of organic compounds (RH), hydroxyl radicals react to form organic radicals (R·). This process, known as chain propagation (Equation 3), begins when hydroxyl radicals oxidize organic compounds (RH) by abstracting a proton, producing highly R·, which can undergo further oxidation(Pilli et al., 2015b).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^- \quad \text{Equation 1}$$

$$OH + Fe^{2+} \rightarrow OH^- + Fe^{3+} \quad \text{Equation 2}$$

$$RH + OH \rightarrow H_2O + R \quad \text{Equation 3}$$

This type of pretreatment is generally applied to increase the dewaterability of sludge. Along with improvement of dewaterability, studies have reported its role in improving the solubilisation of sludge and CH_4 production. Siciliano et al. (2016). studied this process and achieved up to 80% phenol abatement and 90% VFA production using H_2O_2 at a dosage of 0.05 g H_2O_2/g COD in combination with lime. A pretreatment involving 50 g H_2O_2/kg DS and 0.07 g Fe^{2+}/g H_2O_2 at pH 3 resulted in a significant improvement in SCOD, increasing it by approximately 5.0 times, and leading to a 75% higher biogas production compared to the control(Zhen et al., 2017). Pilli et al. (2015b)applied a similar treatment to secondary sludge using 60 g H_2O_2/kg TS, 0.07 g Fe^{2+}/g H_2O_2 at pH 3, and observed a 15% increase in methane yield, from 430 to 496 m^3 CH_4/Mg VS degraded. This technique is appealing due to three key features: (a) the formation of hydroxyl radicals, (b) the low pH, which is comparable to acid pretreatment, and (c) improved dewaterability of the digestate (Gonzalez, 2022b; Neyens et al., 2003a). However, the need for iron salts and other chemical reagents to re-adjust the pH can lead to increased costs and higher energy consumption.

2.5 Bio-hydrogen (Bio-H_2) production

Bio-H_2 production from sewage is emerging as a promising approach to addressing energy and environmental challenges. This process utilizes anaerobic digestion, fermentation, and other biotechnological methods to convert organic matter present in sewage into H_2. H_2, being a clean and high-energy fuel, holds potential for sustainable energy systems, offering a pathway to reduce greenhouse gas emissions and reliance on fossil fuels (Yao et al., 2018b).

Currently, hydrogen production is predominantly dependent on fossil fuels, with approximately 60% derived from dedicated primary hydrogen-producing facilities(Osman et al., 2023a). It is reported that around 71.27% of hydrogen is produced from natural gas (NG), 27.27% from coal, 0.7% from petroleum, and the remaining 0.7% from water electrolysis(Osman et al., 2018, 2023a) . However, hydrogen production from fossil fuel reforming is neither renewable nor carbon neutral, as it involves substantial greenhouse gas emissions.

Figure 7 gives an overview of the various methods by which bio-H_2 can be produced using sewage sludge.

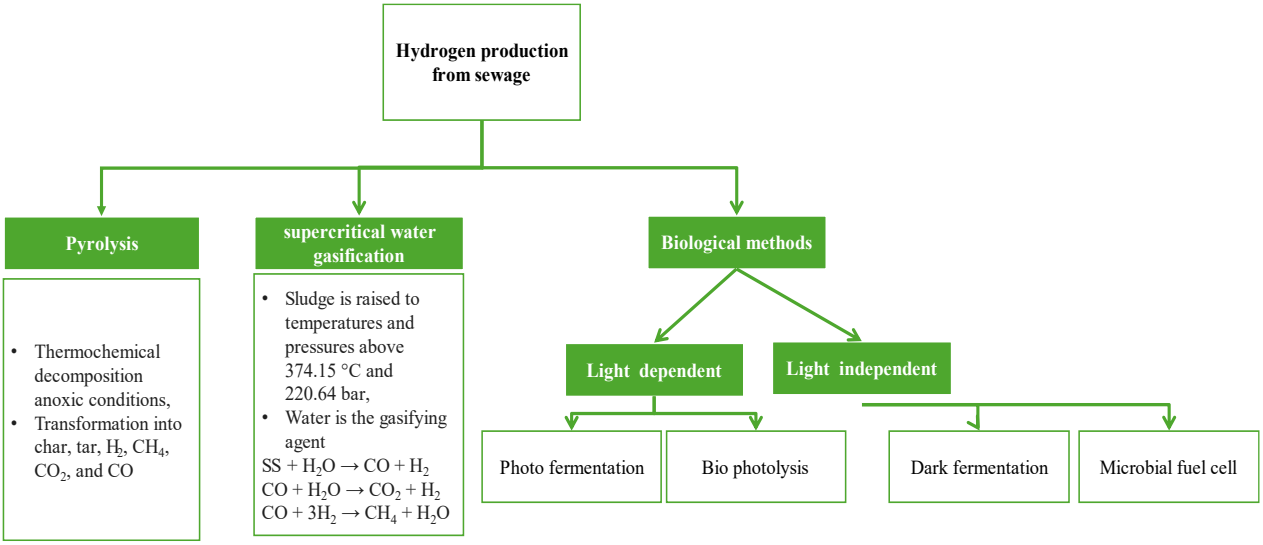


Figure 7 Overview of various methods to produce bio-H_2(Cao et al., 2022b; Kaur et al., 2019; Ram et al., 2024a), SS is sewage sludge

Bio-H_2 through the biological route is sustainable approach to meet future energy demands, given that it is less energy-intensive and sustainable, due to the high organic content of sewage sludge, production of bio-H_2 through sewage is widely researched(Ram et al., 2024a).

Biological methods for H_2 production

The pathway involved in H_2 production can be divided into

1. **Bio fermentation** (dark fermentation and photo fermentation),
2. **Bio photolysis** (direct and indirect),
3. **Bio electrochemical** system such as microbial electrolysis cells (MEC)

Alternatively, these processes can be further classified as shown in figure 7 (based on process conditions with or without sunlight) Figure 8 is an overview of the biological methods used for H_2 production adapted fromRam et al., (2024)

Amongst all the biological methods, the H_2 generation through dark fermentation is more widely used. It is seen as an economical way to produce H_2 from sewage sludge(Yao et al., 2018b).Therefore, dark fermentation offers several advantages such as that it does not require light energy input, has a higher hydrogen evolution rate compared to bio photolysis and photo-fermentation(Ram et al., 2024).It utilizes a wide range of substrates including renewable biomass and organic waste and can be scaled up using existing reactors (Cao et al., 2022b).

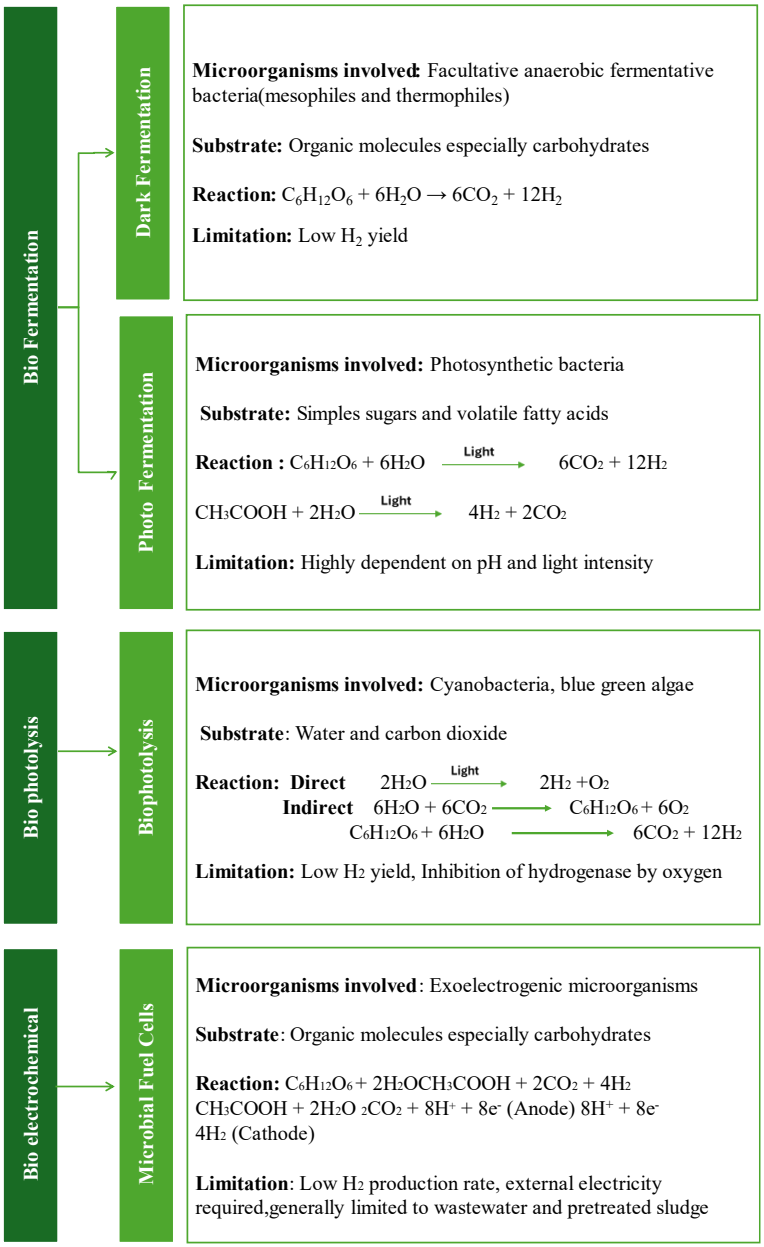


Figure 8 The overview of all the biological processes of H_2 production with participating bacteria

2.6 Dark fermentation

In anaerobic fermentation, microorganisms degrade carbohydrates in the absence of oxygen, leading to the reduction of protons (H^+) by electrons generated from the breakdown of these carbon sources (Ram et al., 2024b). This process ultimately results in the production of molecular H_2. The conversion involves the oxidation of carbohydrates, which generates electrons used to reduce protons, thereby forming hydrogen gas (Ghimire et al., 2017; Tian et al., 2019). This process is primarily facilitated by hydrogenase enzymes, which are commonly found in hydrogen-producing bacteria (HPB).

2.6.1 Role of Hydrogenase Enzymes

Hydrogen production in anaerobic fermentation is catalyzed by two key enzymes: [NiFe]-hydrogenase (NiFe-H_2ases) and [FeFe]-hydrogenase (FeFe-H_2ases). These enzymes are responsible for facilitating the reversible reactions involved in hydrogen metabolism (Yang & Wang, 2019).Among these, FeFe-H_2ases exhibit higher activity in hydrogen production compared to NiFe-H_2ases (Osman et al., 2023b). However, NiFe-H_2ases are generally more sensitive to oxygen, which can inhibit their function (Ghimire et al., 2017). The theoretical yield of hydrogen from glucose is approximately 4 moles of H_2 per mole of glucose. In practice, the actual hydrogen yield is often lower due to the formation of byproducts such as acetic acid, butyric acid, and propionic acid. Typically, the maximum observed H_2 yield during dark fermentation is around 2 moles of H_2 per mole of glucose (Osman et al., 2023b; Tian et al., 2019).

2.6.2 Fermentative Pathways

There are four primary fermentative pathways discussed for H_2 production: the acetic acid pathway, butyric acid pathway, propionic acid pathway, and ethanol pathway. The equations representing these pathways are provided below and have been adapted from van Lier et at. (2020), and Osman et al. (2023b) The application of these pathways is explored in detail within this thesis.

1. **Acetic Acid Pathway:**

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{Equation 4}$$

2. **Butyric Acid Pathway:**

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{Equation 5}$$

3. **Propionic Acid Pathway:**

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + CH_3CH_2COOH + CO_2 + H_2 \qquad \text{Equation 6}$$

4. **Ethanol Pathway:**

$$CH_3CH_2OH + H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad \text{Equation 7}$$

These pathways can operate independently or concurrently, depending on reactor conditions such as pH and the microbial community involved. Usually, acetic acid pathway is obtained as a mixed pathway. Although the propionic acid pathway theoretically exists, it does not produce H_2 on its own but may contribute to H_2 production when involved in mixed fermentative pathways (J. Li et al., 2009). The ethanol fermentation pathway has gained particular attention due to its potential for high H_2 yields (Jianzheng Li, Binling Ai, 2014).

2.6.3 Overview of Metabolism

Dark fermentation involves four types of biochemical reactions: (i) utilization of key enzymes such as pyruvate: formate lyase (PFL) and formate hydrogen lyase (FHL); (ii) reactions involving pyruvate: ferredoxin oxidoreductase (PFOR) and ferredoxin-dependent hydrogenase (HydA); and (iii) the

utilization of NADH for bio-H_2 evolution(Hay et al., 2013). These pathways are facilitated by HPB.The bacteria involved in these processes can be classified into three categories: strict anaerobes, facultative anaerobes, and aerobes (Osman et al., 2023). This study focuses primarily on the roles of strict anaerobes and facultative anaerobes in hydrogen production. Key bacterial species responsible for these processes include *Escherichia coli*, *Enterobacteriaceae*, thermophilic bacteria, *Clostridium species*, and *Ethanoligenens harbinense* .(Osman et al., 2023) Table 2 provides an overview of the types of fermentative pathways, the organisms involved, and their corresponding metabolic processes.

Table 2 The overview of the fermentation pathways the representative bacteria and the metabolic pathways adapted from (Z. Li et al., 2021)

Type of fermentation	**Species**	**Metabolic pathway**	**Key enzyme**	**H_2 yield**	**Type of bacteria**	**Reference**
Ethanol fermentation	*E. harbinense Ethanoligenens B49*	PFOR/FeFe-H_2ase co-metabolic pathway	FeFe-H_2ase, PFOR	2.81 mol-H_2/mol glucose	Strict anaerobe	(Z. Li et al., 2020)
Butyric acid fermentation	*C. butyricum*	PFOR/FeFe-H_2ase synergetic catalytic pathway	FeFe-H_2ase, PFOR, nitrogenase	1.90 mol mol-H_2/mol glucose	Strict anaerobe	(Jenol et al., 2014;)
Mixed fermentation	*E. coli*	PFL and PFOR co-metabolic pathway	Hyd, FDH, PFOR	0.27 mol H_2/mol Glucose	Facultative anaerobe	(Balderas-Hernandez et al., 2020)
Mixed fermentation	*Thermoanaerobacterium*		NiFe-H_2ase	7.04mmol H_2/gm Xylan	Facultative anaerobe	(Jiang et al., 2019)

Ethanol type of fermentation

The biosynthesis of FeFe-H_2ases in *E. harbinense* involves a complex interplay of genes, including structural genes (hyd1-hyd4), which encode the core hydrogenase enzymes critical for H_2 evolution. Additionally, the hydE, hydF, and hydG genes are responsible for the maturation of the H-cluster, a vital component of these enzymes(Z. Li et al., 2021). The organism contains ten ferredoxin-encoding genes, including those for 4Fe-4S and 2Fe-2S ferredoxins, which are key electron carriers that work in tandem

with hydrogenases. These ferredoxins, found across various organisms, include specific domains like the [2Fe-2S] plant ferredoxin-like domain, a unique [4Fe-4S]-containing fold, and a 2[4Fe-4S] domain(H. Huang et al., 2016; Peden et al., 2013).

In addition to these, *E. harbinense* has PFOR genes that facilitate the oxidative decarboxylation of pyruvate to acetyl-CoA. Moreover, this organism possesses at least seven alcohol dehydrogenase (Adh) encoding genes and one acetate kinase (Ack)-encoding gene, which are crucial for ethanol and acetic acid synthesis and metabolism. Collectively, these genomic features suggest a sophisticated ethanol-H_2 co-metabolic pathway within *E. harbinense*, driven by the coordinated action of PFOR and FeFe-H_2ases(Z. Li et al., 2021; Peden et al., 2013).

2.6.4 Operational Parameters Influencing Hydrogen Production

Sewage sludge is highly organic; however, the complex structure prevents the uptake or organic materials for hydrogen producing bacteria(Ananthi et al., 2024). The most widely used option for addressing this challenge is the conducting a pretreatment of sludge. Pretreatment helps to break down the complex structure of the substrate and release the organics which can be taken up by HPB (Ananthi et al., 2024). The pretreatments can be classified into physical, chemical, biological, physico-chemical. Figure 9 give an overview of the commonly used pretreatment technologies for dark fermentation.

Physical pretreatment	Chemical pretreatment	Physico-chemical	Biological Pretreatment
Thermal Grinding, milling, cutting, shearing, and chipping • **Objective :** Reducing the particle size and increase the available surface area.	Organic solvents, acids, alkali, ionic liquids, plasma, and metal chlorides • **Objective:** break the rigid structure of the lignocellulose present.	Steam explosion, subcritical water treatment, and liquid hot water treatment • **Objective:** break the refractory surface of lignocellulosic substances.	fungi addition • **Objective:** enzymatic degradation of lignin, hemicellulose etc

Figure 9 The overview of commonly used pretreatment technologies before dark fermentation of sewage sludge adapted from (Ananthi et al., 2024a)

The various other factors affecting the H_2 production are pH, temperature and the loading rates.

pH

pH is a critical factor influencing H_2 production, impacting both the type of fermentation that occurs and the microbial communities involved. The pH level determines which bacterial species can thrive, thereby either promoting or inhibiting specific classes of bacteria. Various studies have reported different pH ranges as optimal for H_2 production. For instance, some research suggests that a pH range of 5.5 to 6.5 is most conducive to H_2 production (Ananthi et al., 2024b), while other studies advocate

for a slightly more acidic range of 4 to 5.3 as being ideal (Jianzheng Li, Binling Ai, 2014; Ren et al., 1997). Additionally, there are findings that indicate alkaline conditions, with a pH range of 8 to 11, can also be favourable for H_2 production (El-Qelish et al., 2020; Y. Zhao et al., 2010).

Given this diversity of findings, it can be concluded that the lower limit for effective H_2 production is around pH 4, with ideal operating conditions generally falling between pH 4 and 6. The activity of FeFe-H_2ases an enzyme crucial for H_2 production, is particularly sensitive to pH. Very low pH levels (below pH 3) can be detrimental to H_2 production, as the excess protons can infiltrate bacterial cell walls, inhibiting microbial growth and activity (Ananthi et al., 2024b).

Moreover, some literature highlights the benefits of operating within an alkaline pH range (8-11), arguing that higher pH levels not only enhance the hydrolysis of proteins—abundant in sewage sludge—but also support the activity of alkalotrophic microorganisms that produce hydrogen from the resulting amino acids(El-Qelish et al., 2020; Wan et al., 2016a). Additionally, an alkaline environment can inhibit homoacetogens, which are major consumers of H_2 and tend to be acid-resistant(Y. Zhao et al., 2010). The varying opinions on the optimal pH for H_2 production can be attributed to two primary considerations: (i) inhibiting H_2-consuming organisms like methanogens and promoting fermentation pathways, such as the ethanol pathway, which operates optimally around pH 5, and (ii) suppressing homoacetogens while promoting alkalotrophic hydrogen producers (El-Qelish et al., 2020).

Temperature

Temperature plays an important role as it can affect the hydrogen production rates, substrate consumption rates, type of VFA's produced. Table 3 gives an overview of the benefits and disadvantages temperature ranges, mesophilic condition (30 -39°C), thermophilic (50-64°C) and hyper thermophilic (>64°C)(Ram et al., 2024a).

Table 3 The overview of the benefits and disadvantages at the temperature ranges

Temperature range	Type of bacteria	Positives	Negatives	Reference
Mesophilic	Clostridium Enterobacter (*Clostridium beijerinckii, Clostridium butyricum, Enterobacter aerogenes and Enterobacter asburiae*) *Citrobacter*	Widely used as it is economic	Growth of non HPB Bacteria unable to utilize cellulose without external cellulase enzyme	(Ananthi et al., 2024a; d'Ippolito et al., 2010; Ram et al., 2024a)
Thermophilic and hyper thermophilic	*Thermoanaerobacterium thermosaccharolyticum*, *Caldicellulosir, sccharolyticus*	Growth of HPB, elimination of HCB High substrate hydrolysis	Higher energy usage compared to mesophilic Lower volumetric productivity	(Ananthi et al., 2024a; A. Singh et al., 2015) (Hallenbeck, 2009; Ram et al., 2024a)

Organic Loading Rate (OLR) and Hydraulic Retention Time (HRT)

The efficiency of biogas production from organic waste is largely dependent on maintaining balanced reaction rates across the various stages of anaerobic digestion. High OLR can disrupt this balance by causing the excessive accumulation of VFAs, which can lead to the acidification of the bioreactor (Wainaina et al., 2019). When VFAs accumulate beyond the metabolic capacity of methanogenic archaea, these organisms are unable to effectively metabolize the VFAs, leading to a further buildup of acids and a subsequent decrease in pH (Franke-Whittle et al., 2014; Yuan & Zhu, 2016). This pronounced drop in pH inhibits methanogenic activity, exacerbating the imbalance and potentially leading to reactor failure.

One approach to mitigating the detrimental effects of high OLR on the anaerobic digestion process is to operate at reduced HRT in continuous or semi-continuous cultures. HRT is inversely related to the dilution rate (D), which is directly proportional to the maximum microbial growth rate (μmax) (Lu et al., 2011; Valdez-Vazquez & Poggi-Varaldo, 2009). This operational strategy supports the sustained activity of fast-growing microbial populations, such as HPBs, thereby enhancing the potential for H_2 production while minimizing the impact of HCB.

Table 4 provides an overview of the growth kinetics of common HPBs present during dark fermentation, including their maximum specific growth rate (μmax) and substrate affinity (Ks). This table is adapted from CHEN et al. (2006).

Table 4 The overview of the prominent HPB and the growth kinetics

Culture	Mode of operation	Test substate	μ_{max} (h^{-1})	K_s (gCOD/L)	Reference
Citrobacter	continuous	Glucose	0.22	-	(CHEN et al., 2006)
Enterobacter	Batch	Glucose	0.33	3.9	(Kumar, 2000)
Caldicellulosir	Batch	Sucrose	0.13	0.8	(van Niel et al., 2003)
E. harbinense	batch	glucose	0.31	-	(Liu et al., 2015a)
C.butyricum	batch	glucose	0.77	-	
Mixed	continuous	sucrose	0.172	0.068	(Liu et al., 2015b)

Based on μmax values, it can be inferred that continuous hydrogen-producing reactors can be effectively operated at lower HRTs. Studies have demonstrated that a lower HRT is associated with improved H_2 production. For instance, one study reported nearly 7 liters per day of H_2 production at an HRT of 4 hours in a continuous stirred-tank reactor (CSTR) system(CHEN et al., 2006). Similarly, (CHANG, 2002) reported an optimal hydrogen production rate of 0.42 L/h/L in a fixed-bed reactor operating at an HRT of 2 hours. Additionally, the higher Ks values observed in biological fermentation compared to traditional anaerobic digestion suggest that higher OLRs may be more suitable for hydrogen production processes (CHEN et al., 2006).

2.7 Conclusion and Research gap

The current literature indicates that pretreatment can play a role in improving AD. Of the many pretreatment technologies, thermal pretreatment technologies are more widely used in full scale. LTTP is a promising method and has a huge potential to be scaled up.

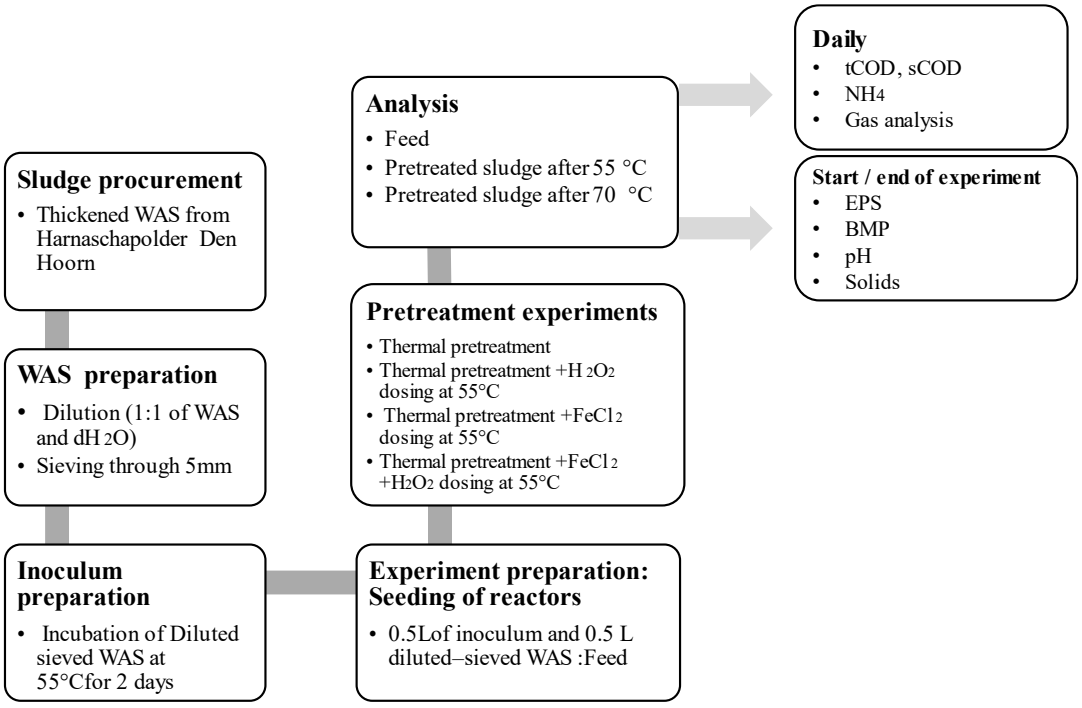
Bio- H_2 production from sewage sludge through biological routes is gaining importance as there is a need for sustainable H_2 production method. Dark fermentation is a widely used biological method Which requires pretreatment of the complex sewage sludge to improve H_2 yield. Therefore, pretreatment can play a vital role in enhancing both the hydrolysis and dark fermentation processes.

Apart from pretreatment, it is indicated by various studies that thermophilic temperature can boost H_2 production. LTTP largely operates in thermophilic temperature bandwidth and therefore, can as good agent for microbial growth and faster conversions. In closed tanks at a thermophilic range, suitable operation mode and organic loading, it can also form a suitable environment for dark fermentation. While scaling up the LTTP technologies, there is a higher chance of gas production at a pretreatment stage. However, this aspect of gas production at the pretreatment stage is not widely reported.

Currently, the pretreatment methods for H_2 production, just focus on improving hydrolysis. However, to improve the H_2 yield, there should be a focus on improving the metabolic pathways, combining various parameters like temperature, pH which can facilitate the growth of HPB along with hydrolysis. This aspect is still not widely reported in literature. Therefore, the research questions and the research focus for this thesis (section 1.2) was formulated to address these gaps.

3.Materials and Methods

The following section gives a detailed overview of the various experimental methods and procedures followed throughout the thesis. This section is further divided into sourcing of Waste Activated Sludge (WAS), pretreatment experiment preparation, analytical methods. Figure 10 is a schematic giving an overall view of the methodology followed.



dH_2Odistilled water, the diluted-sieved WAS was fed to the reactor during the experiments: Feed.,tCOD: Total Chemical Oxygen Demand, sCOD: soluble Chemical Oxygen Demand, EPS: Extracellular Polymeric Substances, BMP : Biomethane Potential

Figure 10 The methodology followed throughout the thesis

3.1 Sourcing of the Waste Activated Sludge (WAS)

The WAS used throughout the study was the secondary sludge obtained after thickening from Harnaspolder Den Hoorn, Netherlands. This was chosen to replicate the input characteristics of Themista. The sludge was collected weekly from the sludge thickener and store at 4°C. Sludge was collected before each set of experiments.

3.1.1 WAS Preparation

WAS was mixed with an equivalent amount of distilled water to achieve a 1:1 ratio, enhancing the sludge's flow properties through the reactor system. The diluted sludge was then sieved using a 5 mm

mesh to remove larger particulates. The diluted and sieved sludge was stored at 4°C and acclimatized before use.

3.1.2 WAS Inoculum Preparation

The sieved and diluted sludge was further processed to prepare the inoculum. 1L of diluted-sieved sludge were placed in an Eppendorf® New Brunswick™ Innova® 42/42R Incubator Shaker set at 55°C and 200 RPM for 2 days.

3.2 Pretreatment experiment preparation

3.2.1 Experimental setup

The prepared inoculum was combined with an equal amount of diluted-sieved WAS. Each reactor bottle was filled with 1L of this mixture and placed in water baths maintained at 55°C and 70°C. The bottles were connected to Watson Marlow 323 series peristaltic sludge pumps. Initially, the pumps were operated at maximum RPM to expel air from the tubing, followed by adjustment to maintain a flow rate of 136 mL/min. After flow stabilization, the water baths switched on to achieve the required experimental temperatures. Subsequently, rotators and sample points were connected. Gas bags were flushed with N_2 gas and purged to remove any residual O_2 before being connected to the system. The head spaces were where tightly sealed with a rubber seal with two holes. Sampling point was connected to one hole meanwhile gas line was connected to the ritter meter (RITTER MilliGascounter) and then connected to their respective gas bags. Then the rotators were connected. For the Thermo-chemical experiments, after the cleaning of gas bag peristaltic chemical pumps (Watson and Marlo 120 series) were connected to the system. Before connection, the chemical lines are filled with the chemicals so that no air enters the head space. Finally, the timers for the sludge pumps and the chemical pumps were connected. The timers turned on, in an interval of 1.5hrs to maintain the HRT at 5.5 hrs, each time the sludge pumping was done for 2 mins, pumping 136mL/min. The HRT of the system was maintained to be 5.5 hrs at 55°C and at 70°C while in the full scale the HRT at 70°C was 3.5 hrs. This was done simplify the operation of the lab scale setup.

3.2.2 Sampling Time and Duration

After the operation started, two samples were taken in the day one in the morning (9:15), afternoon (13:45). The total experiment duration was 3.8 days the first sample was collected at 0.8 day after the system startup. The reactors were semi-continuously fed with untreated diluted-sieved WAS (also referred to as untreated WAS in this manuscript) over the operation period Sampling was conducted prior to the onset of each pumping cycle.The interval between these two samples approximates a complete HRT. This was done to ensure that sample process could be done the same day. Gas samples were extracted from the gas bag, which was connected to a syringe. The syringe was purged with the gas three times before collecting the sample, ensuring the removal of residual air. Each gas sample comprised 10 mL.

Sludge samples were obtained using a syringe connected to the sample port on the top of each reactor. The syringe was flushed with sludge three times to ensure the collected sample originated from the reactor and not from residual tubing contents. Each sludge sample volume was 10 mL. Figure 11 is the schematic of the experimental setup at TU Delft.

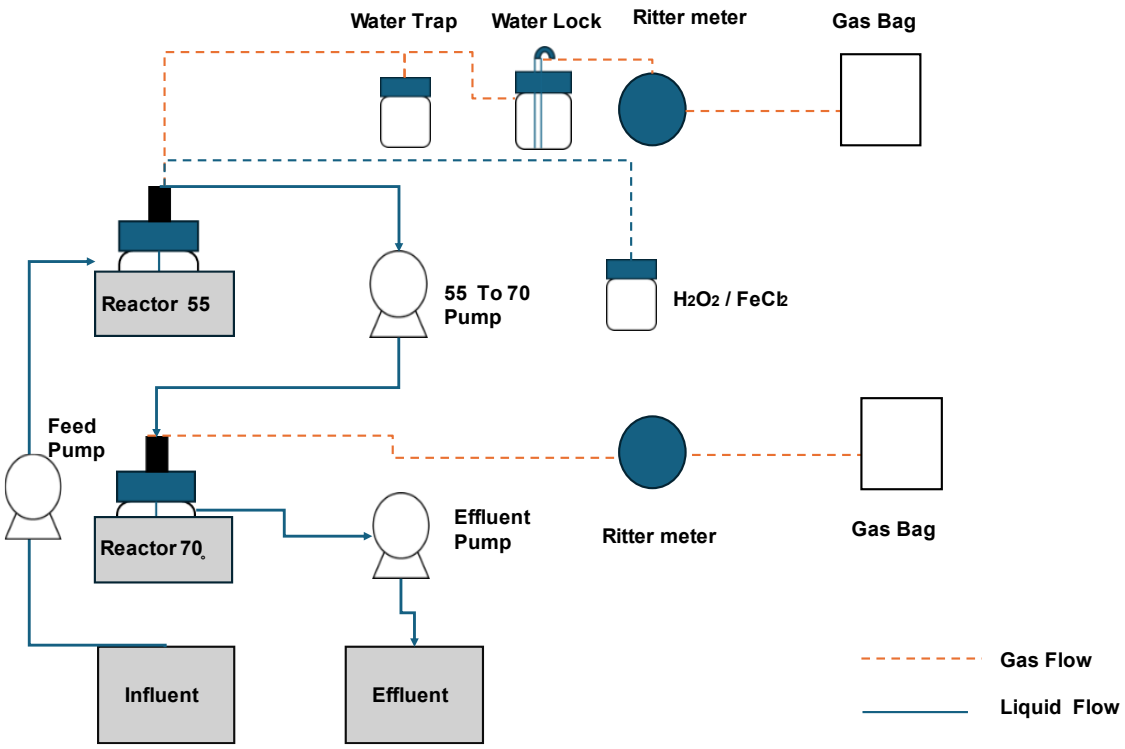


Figure 11 The schematic of the experimental setup of the of the thermal, thermo-chemical pretreatment systems at TU Delft

3.2.3 Chemical additions

Hydrogen Peroxide (H_2O_2)

H_2O_2 with a concentration of 30% w/w (Merck, U.S.A.) was used. A dosage of 15 mg/gTS was selected, based on the dosage used for Themista. The application strategy or sequence of H_2O_2 addition significantly affects pre-treatment outcomes (Gonzalez, 2022).To align with this understanding, H_2O_2 was added 30 minutes after sludge pumping. This timing strategy, consistent with (Y. Wang et al., 2009) and is based on the rationale that pre-incubation at 55°C would denature catalase, which is an enzyme present in cells, which rapidly breaks down H_2O_2 into H_2O and O_2 to protect the cells from the toxic effects of H_2O_2, ensuring its inactivity before the addition of H_2O_2. It was also to ensure that the temperature was maintained at 55°C at the time of dosing.

Ferrous Chloride tetrahydrate ($FeCl_2.4H_2O$)

$FeCl_2.4H_2O$ (98%, Thermo Scientific Chemicals) was administered at a dosage of 15 mg/gTS, a standard practice dose which is similar to the dosage for H_2O_2 addition in this study. This chemical was introduced 30 minutes post-sludge pumping like the strategy for dosing H_2O_2.

Combined Ferrous Chloride tetrahydrate and H_2O_2 Treatment

For combined treatment, both $FeCl_2.4H_2O$ and H_2O_2 were administered at 15 mg/gTS each. The $FeCl_2.4H_2O$ was added first, followed by H_2O_2 two minutes later. This staggered addition was employed to minimize pre-mixing and ensure effective treatment within the reactor system. The chemical dosing was done 30 mins after sludge pumping.

3.4 Analytical methods

The sludge sample collected were divided into two, one is the total sludge sample and the soluble sample. For soluble sample preparation, the collected sludge samples were transferred into 2 mL Eppendorf tubes and centrifuged at 14,000 g for 5 minutes. The resulting supernatant was decanted into 10 mL centrifuge tubes. Thermal pretreated samples were diluted twofold with distilled water, while thermo-chemical samples were diluted fourfold. These diluted samples were then filtered through a 0.45 µm syringe filter to remove any remaining organic material.

3.4.1 Chemical oxygen demand (COD), Ammonium (NH_4^+) Analysis

Total and soluble COD (tCOD, sCOD respectively) were measured using LCK 014 and 514 kits (Hach Lange, Germany) respectively and a HachDR3900 spectrophotometer. NH_4^+ was analysed with LCK 303 (Hach Lange, Germany).

3.4.2 Solids Analysis

Total solids (TS), volatile solids (VS), total suspended solids(TSS), and volatile suspended solids(VSS) were measured gravimetrically by the procedure given in 2540 G and 2540 D APHA standard method respectively(APHA standard methods, 2013)

3.4.3 Volatile fatty Acids (VFA)

The VFA were measured by adding 700µl of soluble sample was added to a VFA vial then 700 µl of 1-pentanol was added. Finally, a 10 µl of formic acid was added to make a working volume of 1. 5ml.The samples were analysed at gas chromatography with a Flame Ionisation Detector (FID) (Agilent 7890A, USA) and a column (Agilent 19091F-112). Helium was used as carrier gas (1.8 mL/min); injection port and oven temperatures were 240 °C and 80 °C, respectively.

3.4.4 pH measurement

pH was measured with a SenTix 940 IDS probe (WTW, Germany).

3.4.5 Gas Composition and quantity analysis

The gas composition was measured by the Micro GC (Varian CP 4900, USA) with thermal conductivity detector (TCD) and columns, i.e. Mol-Sieve-5A-PLOT and argon as carrier gas (1.47 mL/min, 80 °C)

PoraPlot-U and helium as carrier gas (1.47 mL/min, 65 °C). The gas sample was injected into the GC from the injection point. The percentages of N_2, H_2, CH_4, CO_2 and CO were measured.

3.4.6 The Biomethane Potential (BMP)

BMP was measured in triplicates using the AMPTS-2 system (Bioprocess Control, Sweden) following the procedure by Holliger et al. (2016). The test was conducted in triplicate in 500 mL bottles filled with 240ml of inoculum (the anaerobic digestate from Harnaschpolder WWTP and the corresponding substrate (non-treated WAS, 70°C treated WAS,). The bottles were filled for a total volume of 400 ml of an Inoculum substrate ratio (ISR) of 2 on VS basis was maintained. One Blank triplicate was also run to account for the endogenous methane production of the inoculum, keeping the same ISR as for the WAS substrates. Macro- and micronutrients and Bi-carbonate buffer were added (12.2 mL of 0.2 M $K_2HPO_4 \cdot 3H_2O$; 7.8 mL of 0.2 M $NaH_2PO_4 \cdot 2H_2O$; 2.4 mL solution; 170 g/L NH_4Cl, 8 g/L $CaCl_2$. $2H_2O$, 9 g/L $MgSO_4$. $7H_2O$; 0.24 mL and 8mL respectively). The head space was purged with N_2 gas for 30 seconds. After that, the bottles were incubated at 35°C with a mixing of 150rpm in New Brunswick Innova® 43/43R. The biogas then produced passed through 3M NaOH scrubber solution that absorbs CO_2. The analysis was continued till an asymptotic behaviour was obtained in the graphs.

3.4.7 Extracellular Polymeric Substance (EPS)Extraction Method

The thermal EPS extraction method utilized in this study was adopted and modified from the procedure followed by Cabeza et al. (2023)for the extraction of soluble EPS (S-EPS), loosely bound EPS (LB-EPS) followed by the tightly bound EPS (TB-EPS) surrounding the cells.

The key steps:

1. **Centrifugation:** The initial step involved centrifuging the sludge suspension at 14000 g for 15 minutes in 50 mL tubes to dewater the sludge. The supernatant was collected as the S-EPS fraction.
2. **Resuspension and Extraction of LB-EPS:** The sludge pellet was resuspended in 50 mL of 0.05% NaCl solution. This NaCl solution was pre-heated to 70°C to warm the sludge suspension to 50°C. The sludge was then mixed in a vortex mixer for 1 minute, followed by centrifugation at 14000 g for 15 minutes. The resulting supernatant was designated as the LB-EPS fraction.
3. **Extraction of TB-EPS:** The remaining pellet was again resuspended in 50 mL of 0.05% NaCl solution. This time, the sludge mixture was heated to 60°C for 30 minutes in a water bath, and subsequently centrifuged at 14000 g for 15 minutes. The new supernatant obtained represented the TB-EPS fraction.

3.4.8 EPS Analysis

The analysis of extracted EPS involved the measurement of proteins, humic substances, and carbohydrates. The proteins and humic substances were quantified using a modified Lowry method(OliverH. Lowry, 1951), whereas carbohydrates were measured using the phenol-sulfuric acid method

3.5 Quantitative calculations

The Biochemical Methane Potential (BMP) is a critical parameter defined as the maximum volume of CH_4 produced per gram of VS substrate The BMP was calculated according to the AMPTS II manual (Bioprocess Control, 2016) through equation 8

$$BMP = \frac{(Vs-Vb)}{(mVs,ss)} \quad \text{Equation 8}$$

Vs- is the accumulated volume of methane produced from the reactor with sample (mL)

Vb - is the mean value of the accumulated volume of methane produced by the three blanks(mL)

mVs,ss - is the amount of organic material (i.e., volatile solids) of substrate contained in the sample bottle(g).

3.6 Statistical analysis

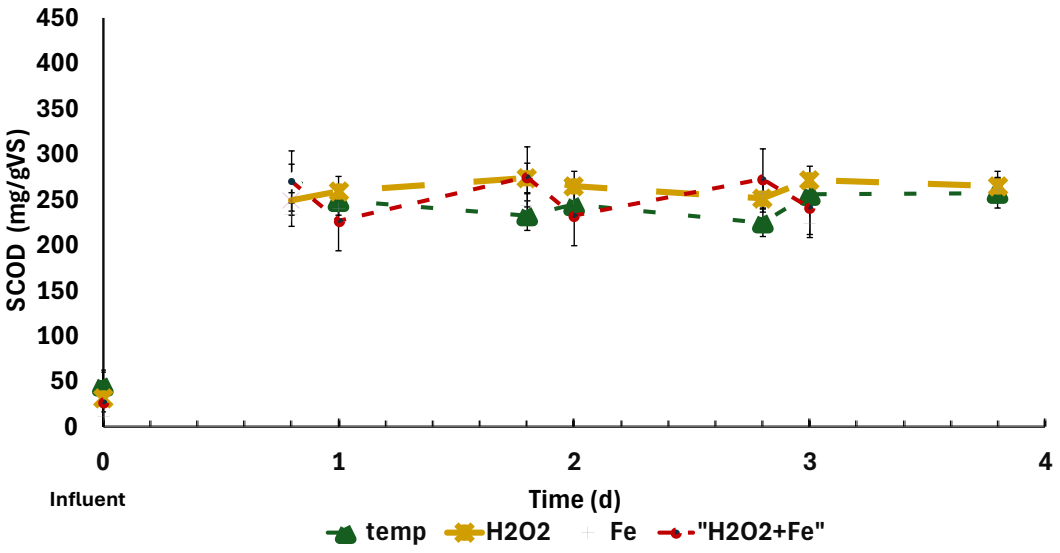
Statistical analyses were done to find the significance between the sCOD release between the pretreatment conditions. One way ANOVA was done using R software (version 4.0.2) with a threshold significance of ($p<0.05$). The significant test for the soluble protein, soluble humic substances and BMP were done t-student test using MS Excel.

4. Results and discussion

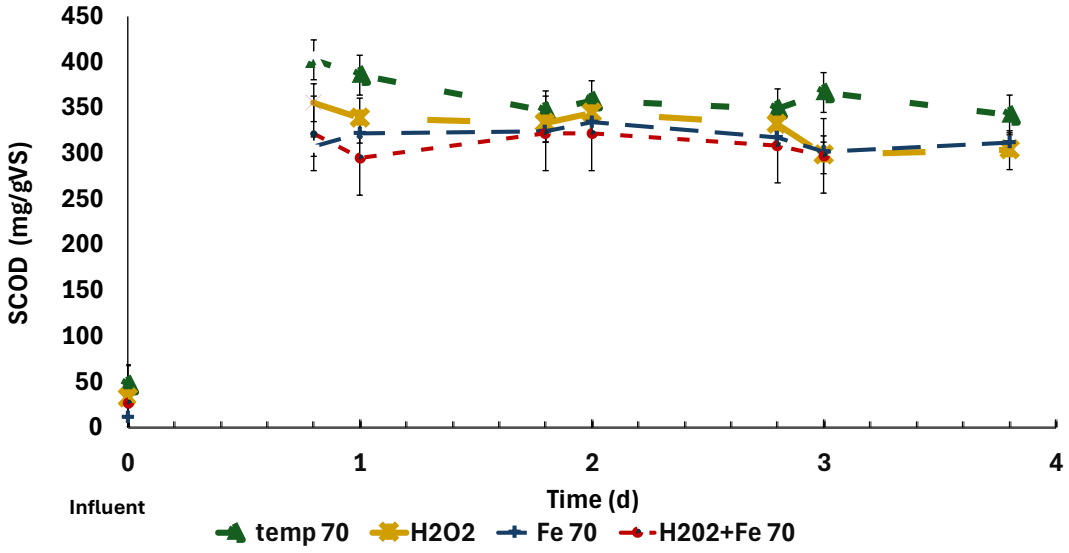
This chapter focuses on the experimental outcomes and the effects of the pretreatment conditions on key parameters, including sCOD release, NH_4^+ release, EPS release, fermentative products, gas composition, and the BMP. Additionally, this section covers supplementary experiments conducted to further substantiate the results and provide a deeper understanding of the processes involved. The overall discussion section aims to elucidate the broader impact of these parameters and assess their implications for a full-scale setup.

4.1 Effect of different pretreatment conditions soluble Chemical Oxygen Demand release(sCOD)

sCOD serves as an indicator of the breakdown of insoluble organic matter into soluble components, reflecting the extent of cell lysis(Xue et al., 2015a). This is illustrated in Figures 12a and 12b, which depict the impact of the various pretreatment methods on sCOD release at 55°C and 70°C, respectively. From figure 12a it can be observed that all pretreatment conditions led to a noticeable increase in sCOD compared to untreated WAS.



(a)



(b)

Figure 12 sCOD release (mgsCOD/gVS) versus time observed over a 16.5-cycles after pretreatment at two temperatures: (a) 55 °C and (b) 70 °C, n=2.Time 0 is the influent sample. The influent/untreated WAS was fed throughout the experiment semi-continuously.

The net changes in sCOD for the different pretreatment methods tested from 0.8 to 3.8 days are as follows: thermal pretreatment at 55°C : -16 ± 16 mg sCOD/gVS; thermal pretreatment with H_2O_2 at 55°C : 16 ± 9 mg sCOD/gVS; thermal pretreatment with $FeCl_2$ at 55°C : 28.5 ± 13 mg sCOD/gVS; and thermal pretreatment with both $FeCl_2$ and H_2O_2 at 55°C : -28.9 ± 35 mg sCOD/gVS.

This aligns with findings reported in the literature(Appels et al., 2010a; Kumar Biswal et al., 2020; Nazari et al., 2017; Xue et al., 2015). This process can be driven by the chemical bond breakage occurring during thermal and thermo-chemical treatment, which includes the degradation and hydrolysis of polysaccharides and proteins (Kumar Biswal et al., 2020a; Mohammad et al., 2024a).

The current study shows a relatively stable trend in sCOD release between 0.8 and 3.8 days of operation. In contrast to previously known studies, such as Xue et al. (2015b), reported a rapid rise in sCOD during the first 48 hours of LTTP at 60°C, followed by stabilization over the next 48 hours in a batch mode. Similarly, Kumar Biswal et al. (2020a) observed a fourfold increase in sCOD within the first 30 minutes of LTTP at 60°C, with a further 50% increase after 3 hours, also in batch mode. This stability observed in this study could be attributed to the semi-continuous mode used in this study, where sludge is periodically added and withdrawn, creating consistent conditions conducive to steady microbial activity and chemical reactions.

Figure 12b indicates that sCOD release is slightly higher at 70°C across all pretreatment conditions compared to 55°C.

This trend is also confirmed by the net increases reported in Table 5, which provides a comprehensive overview of the average increase in sCOD for the different pretreatment conditions at both 55°C and 70°C over 3.8 days.

Table 5 The average increase in sCOD for the different pretreatment conditions observed over a 16.5-day cycle at both 55°C and 70°C

Experiment	**Average sCOD release (mgsCOD/gVS) 55 °C**	**Average sCOD release (mgsCOD/gVS) 70 °C**
Temperature	249±16	364 ± 22
Temperature with H_2O_2	236 ± 9	330 ± 21
Temperature with $FeCl_2$	245 ± 13	317 ± 11
Temperature with H_2O_2 and $FeCl_2$	253 ± 22	311± 12

The higher release of sCOD at 70°C may be due to enhanced solubilization of proteins, carbohydrates, and humic substances, as 70°C is near the temperature at which many proteins begin to denature(A.D. Alber., 2012). This aligns with trends observed in the literature (Dhar et al., 2011a; Kumar Biswal et al., 2020c; Nazari et al., 2017c).

However, the differences in sCOD release among the various pretreatment methods—thermal alone, thermal with H_2O_2, thermal with $FeCl_2$, and thermal with both H_2O_2 and $FeCl_2$—were not statistically significant, as shown by a one-way ANOVA test (p-value $\geq$ 0.05). Although thermo-chemical pretreatments are designed to break down cell structures and increase COD release, it is possible that with extended treatment times, the organic compounds may further get mineralised into inorganic compounds. This phenomenon, discussed in studies by Gonzalez et al., (2018); Kobayashi et al., (2009); Manickam et al., (2014), might explain the similar COD release patterns observed across both thermal and thermo-chemical pretreatments.

4.2 Effect of different pretreatment conditions on Extracellular Polymeric Substance (EPS) release

EPS are metabolites that play a crucial role in the functioning of microorganisms. EPS primarily consist of macromolecules, including proteins, polysaccharides, nucleic acids, humic substances, and lipids (Wu et al., 2023). Proteins and carbohydrates form 70-80% of the EPS in WAS (Neyens et al., 2004). Disrupting the EPS network is crucial for enhancing AD, making cell contents accessible to microorganisms (Dhar et al., 2011b Nielsen et al., 2011). In this study. Proteins (P), carbohydrates(C) and humic substances (HS) have been measured. These have been further categorized into soluble(s-EPS), tightly bound (TB-EPS), and loosely bound (LB-EPS) fractions Figure 13 shows the EPS profile measured for proteins (Figure 13a), humic substances (Figure 13b) and carbohydrates (Figure 13c), before and after pretreatment at 55°C and 70°C.

The overall trend observed is that all pretreatment conditions facilitated a shift from the tightly bound fraction to relatively loosely bound substances in proteins, carbohydrates, and humic substances.

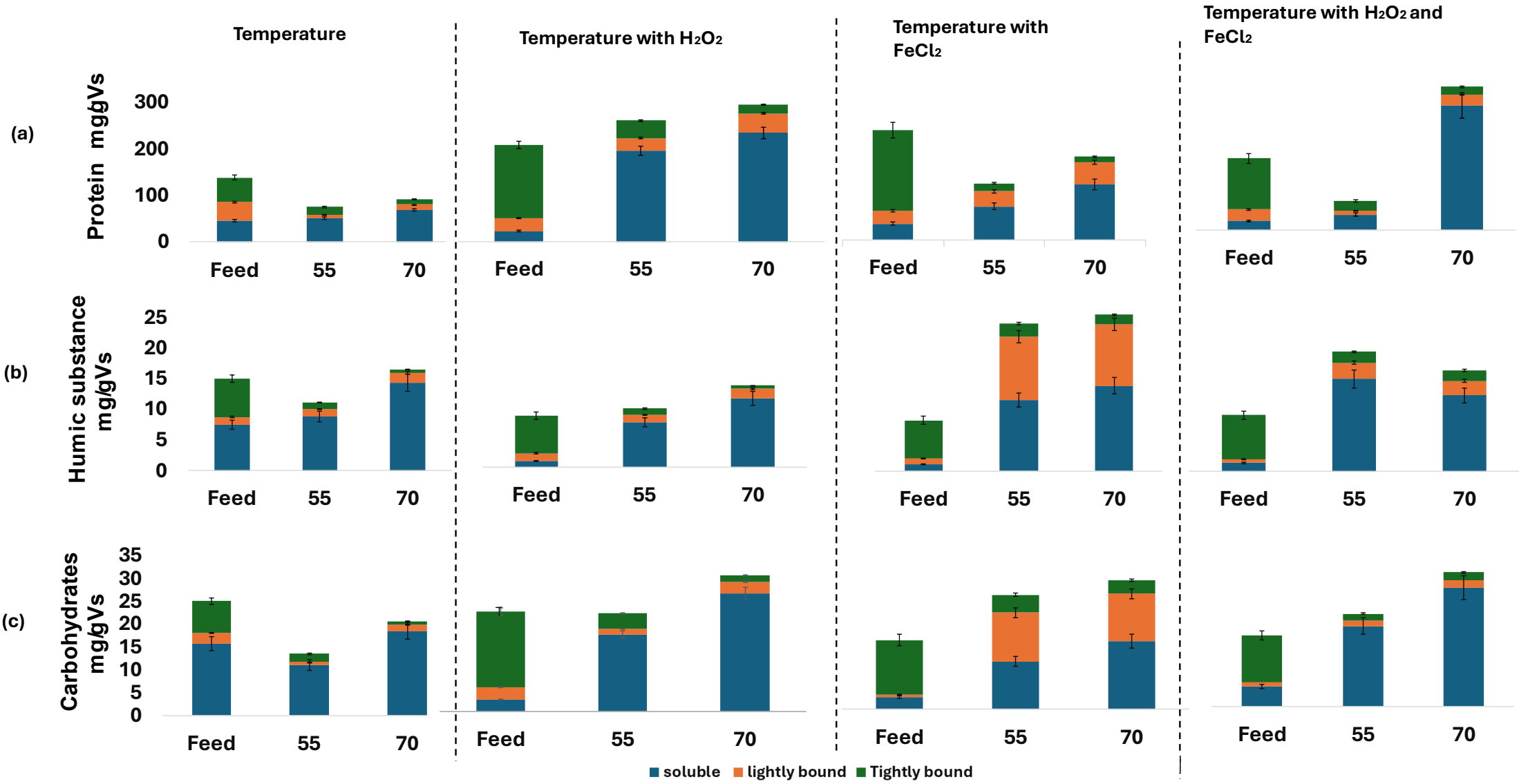


Figure 13 The EPS release in mg/gVS measured before and after pretreatment at 55°C and 70°C measured for the sample taken on 3.8-day, n=3

4.2.1Proteins

It was observed that both thermal and thermo-chemical pretreatments at 55°C and 70°C resulted in an increase in the soluble fraction of proteins compared to non-pretreated sludge (figure 13a). To further elucidate this conversion to the soluble fraction, the percentage of s-protein relative to the total protein content for each pretreatment condition at both 55°C and 70°C was calculated. Additionally, the net increase in the soluble fraction between untreated and pretreated WAS was determined, and the significance of these differences was assessed using the t-test, as presented in Table 6.

Table 6 The overview of percentage of soluble fraction in the sample after pretreatment at 55 °C and 70°C, the net increase in soluble fraction after pretreatment and significance of the increase

Experiment	% Soluble protein (55°C)	% Soluble protein (70°C)	Net increase at 55°C (mg P/gVS)	Net increase at 70°C (mg P/gVS)	Significance after 55°C	Significance after 70°C
LTTP	67	75	4.9 ± 6	22.8 ± 4	$p>0.05$	$p>0.05$
LTTP with H_2O_2	75	79	132.6 ± 3	162.3± 2	$p<0.05$	$p<0.05$
LTTP with $FeCl_2$	59	67	36.4 ± 1	82.5 ± 2	$p<0.05$	$p<0.05$
LTTP with $FeCl_2$ with H_2O_2	52	87	12.7± 8	241.3± 25	$p>0.05$	$p<0.05$

Significance after 55°C: The significance test was conducted between the soluble protein content between the untreated WAS and soluble fraction pretreatment at 55°C; Significance after 70°C: The significance test was conducted between the soluble protein content between the untreated WAS and soluble protein fraction after pretreatment at 70°C.

From Table 6, it is evident that post-LTTP, the percentage of s-protein in the total protein exceeds 50% at 55°C and 60% at 70°C across all pretreatment conditions. This indicates that the pretreatments facilitated a shift from TB- protein to more soluble fractions, suggesting that cell lysis occurred during treatment. The release of intracellular proteins from TB to s-protein indicates towards the effectiveness of these treatments in promoting cell disruption and. This finding is consistent with reports in the literature, where similar shifts in protein solubility following pretreatment have been documented (Dhar et al., 2011b; Kumar Biswal et al., 2020a; Nazari et al., 2017d).

For the thermo-chemical pretreatment experiments, the condition involving thermal treatment combined with H_2O_2 showed a s- protein content exceeding 70% at both 55°C and 70°C. This can be attributed to the oxidative properties of H_2O_2, which likely contribute to the breakdown of cell walls, thereby releasing intracellular materials such as proteins. Notably, the highest s-protein percentage was observed in the condition with thermal treatment combined with $FeCl_2$ and H_2O_2, reaching 87% at 70°C. However, the percentage was significantly lower at 55°C, only 52%. This disparity may be due to the

temperature-enhanced catalytic reaction between Fe and H_2O_2 at higher temperatures, which leads to more extensive protein decomposition, as reported in the literature (Neyens & Baeyens, 2003; Pilli et al., 2015b; Siami et al., 2020). However, the possibly of an error in measurement at 55°C for this condition cannot be ruled out. Conversely, the lower soluble protein content observed in the thermal with $FeCl_2$ condition at both 55°C and 70°C could be attributed to the formation of Fe-organic material complexes that may exhibit reduced solubility, although the precise mechanism remains unclear.

The statistical analysis revealed that, for thermal pretreatment alone, the increase in soluble protein content was not significant compared to untreated WAS, and higher temperatures did not notably enhance this effect. However, for all thermo-oxidative processes, the increase in s-protein was significant at both 55°C and 70°C, except for the thermal with H_2O_2 and $FeCl_2$ condition at 55°C. These findings cautiously suggest that thermo-oxidative pretreatments may offer a distinct advantage over thermal pretreatment alone in facilitating cell lysis and enhancing protein solubility.

4.2.2Humic substances

From figure 13b it can be observed that all pretreatment conditions led to an increase in s-humic substances. Table 7 provides the net increase in humic substances across the different pretreatment methods and the significance of these increases, calculated using a t-test, comparing feed sludge to pretreated sludge at 55°C and 70°C.

Table 7 The net increase in soluble fraction after pretreatment at 55 °C and 70°C and significance of the increase

Experiment	Net increase at 55°C(mgHS/gVS)	Net increase at 70°C(mgHS/gVS)	Significance at 55°C	Significance at 70°C
LTTP	1.4± 0.3	6.9 ± 0.3	$p>0.05$	$p<0.05$
LTTP with H_2O_2	6.5 ± 0.3	10.4 ± 0.3	$p<0.05$	$p<0.05$
LTTP with $FeCl_2$	9.3 ± 0.3	11.4 ± 0.3	$p<0.05$	$p<0.05$
LTTP with H_2O_2 and $FeCl_2$	12.2 ± 0.3	9.8 ± 0.3	$p<0.05$	$p<0.05$

As shown in Table 7, thermal pretreatment resulted in the smallest net increase (compared to the other pretreatments) in humic substances at both 55°C and 70°C. The increase was statistically significant for all conditions except thermal pretreatment at 55°C. There are limited studies, which often focus on humic substance release at temperatures below 70°C. Gonzalez et al. (2018) noted that LTTP generally does not affect humic substances, maintaining stability across a temperature range from 25°C to 80°C. This is consistent with our findings, where no significant increase in the soluble fraction of humic substances was observed after treatment at 55°C. However, not consistent with the observation noted at 70°C. In this study a slight increase was noted after pretreatment at 70°C, as similarly reported by (Deiana, 2019.).

Thermo-oxidative processes showed a higher net increase in humic substances, with the most significant rise observed in the combination of 55°C with $FeCl_2$ and H_2O_2. The powerful oxidizing effects of this combination likely led to the destabilization of the EPS structure, releasing the humic-like substances over time (Guan et al., 2018). This is evident from the increased soluble fraction of humic substances at both 55°C and 70°C.

Research on the interaction between Fe(II) and humic substances post LTTP is limited. However, existing studies suggest that Fe (II) can form stable complexes with humic substances, particularly at a pH around 5 (Gerke, 2021). Although pH was not measured during EPS extraction and analysis in this study, but it was recorded to be around 5 after LTTP with $FeCl_2$ at both 55°C and 70°C. This suggests the likely formation of Fe-Humic complexes. The significant increase in the loosely bound fraction supports this hypothesis. The multivalent cation bridging theory may explain these findings, where negatively charged groups on EPS attract multivalent cations like Fe, which then bind to humic substances and carbohydrates (Oikonomidis et al., 2010). It is possible that Fe-Humic complexes formed in the tightly bound fraction but were extracted as part of the loosely bound fraction.

The literature offers differing perspectives on the impact of humic substances on AD. Some studies report that humic substances enhance enzymatic hydrolysis and improve the hydrolytic enzyme activity (Liu et al., 2015b; Tang et al., 2010). Others note improved acidogenic efficiency due to enhanced electron transfer, with humic substances acting as catalysts (J. Li et al., 2019; S. Wang et al., 2022). However, Huang et al.(2021b); Xu et al. (2020) observed a decrease in protease activity at higher concentrations of humic substances, with a 50% reduction in the acidification rate when humic substances increased from 1 to 2.5 HS/gVS.

The impact of humic substances on AD is influenced by various factors such as concentration, functional groups, sludge characteristics, and microbial community (F. Huang et al., 2021b). Due to the complex and varied nature of humic substances, characterized by diverse physicochemical properties, this study did not perform a detailed analysis of their specific characteristics. Specifically, the nature of the soluble fraction of humic substances and the presence of biodegradable or non-biodegradable organics were not assessed. As a result, the actual impact of the increase in s-humic substances observed in the study on AD, cannot be accurately quantified.

4.2.3Carbohydrates

Figure 13c shows a substantial overall increase in the soluble fraction of carbohydrates before and after pretreatment, with the exception of thermal pretreatment at 55°C. Notably, all pretreatment conditions at 70°C resulted in a higher release of s-carbohydrates. Thermo-chemical pretreatments, particularly thermal with H_2O_2 and thermal with $FeCl_2$ and H_2O_2, exhibited a higher release of soluble carbohydrate fractions compared to thermal pretreatment alone. This shift from tightly bound to loosely bound fractions, is well documented in the literature(Appels et al., 2010; Kumar Biswal et al., 2020a).

Carbohydrates are primarily located within exopolymers, which decompose when disrupted. Oxidative pretreatments, designed to break down cells, also destroy these exopolymers, releasing carbohydrates into the liquid fraction(Dhar et al., 2011b). The greater release of soluble carbohydrates at 70°C compared to 55°C may be due to temperature-assisted partial solubilization of cellulose, as suggested by Kumar Biswal et al. (2020a). This trend has also been observed in other studies (Appels et al., 2010; Nazari et al., 2017; Uma Rani et al., 2012)

For LTTP, the soluble fraction of carbohydrates showed minimal difference between non-pretreated and pretreated WAS. An unusually high soluble fraction of carbohydrates was recorded in the untreated WAS, which may be attributed to a measurement error.

A higher loosely bound fraction was observed in the thermal with $FeCl_2$ pretreatment at both 55°C and 70°C. This parallels the observations made with humic substances, suggesting the potential formation of Fe-organic complexes.

4.2.4 Interrelation of EPS results

This study indicates that all pretreatment conditions facilitated a shift from tightly bound fractions to loosely bound and soluble fractions for proteins, humic substances, and carbohydrates. More than 50% of the protein content was found in the soluble fraction after pretreatment, suggesting that cell lysis occurred, releasing intracellular proteins (Nazari et al., 2017). It was also observed that protein release was higher than carbohydrate release. Although carbohydrates generally decompose more easily than proteins, proteins exhibit greater solubility (Kumar Biswal et al., 2020a). Carbohydrates, which are part of the cell exoskeleton, are released first upon cell breakage, while proteins are mainly present inside the cell. The decomposition of EPS and subsequent cell lysis lead to protein release(Nazari et al., 2017). This trend of higher protein release compared to carbohydrates is supported by numerous studies (Appels et al., 2010; Kumar Biswal et al., 2020; Xue et al., 2015).

It can be hypothesized that the thermal and thermo-chemical pretreatments led to the formation of complex, less biodegradable organic matter. At the applied temperatures with chemicals, a reaction between soluble carbohydrates and/or soluble proteins may have occurred, leading to the formation of Amadori compound type arrangements. This hypothesis is supported by the browning observed in the liquid phase / supernatant of the sludge, a phenomenon similarly reported Appels et al. (2010) and Liao et al. (2016b)where sludge supernatant turned brown after LTTP.

The increase in the loosely bound fraction of humic substances and carbohydrates after pretreatment of thermal with $FeCl_2$ may suggest the adsorption of Fe (II) into the sludge. However, no measurement was conducted to assess the Fe (II) content in the sludge.

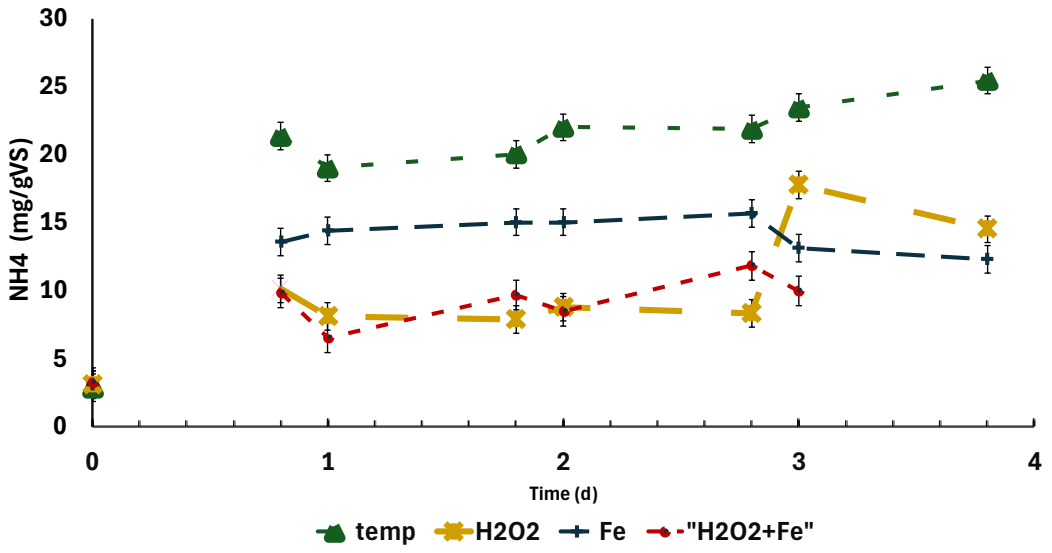
Finally, by considering the COD of 1 mg protein and 1 mg carbohydrate, the theoretical COD release was calculated and compared with the average sCOD release for 55°C and 70°C from section 4.1, the higher sCOD release observed at 70°C can be explained by the higher release of proteins and carbohydrates. (detailed calculations can be found in the Annexure 1)

4.3Effect of different pretreatment conditions on Ammonium (NH_4^+) release

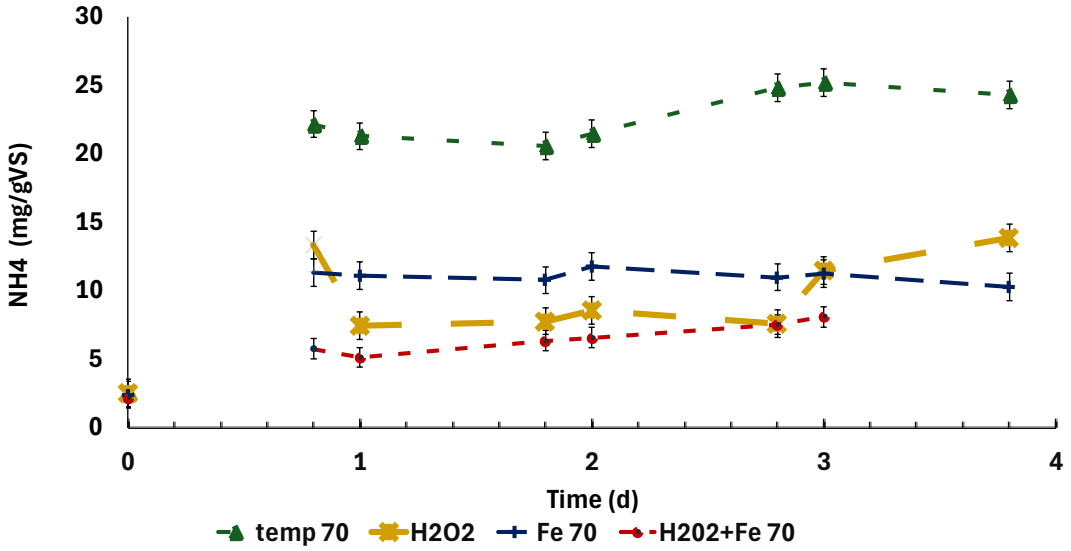
NH_4^+ release during sludge treatment is predominantly attributed to the decomposition of nitrogen-rich compounds, particularly proteins and amino acids (Kumar Biswal et al., 2020a). The release of NH_4^+ and sCOD can serve as a vital indicator for assessing the extent of organic matter solubilization within the sludge matrix (Negral et al., 2015). Figure 14 provides a comparative analysis of NH_4^+across various pretreatment conditions, with Figure 14 a illustrating the result at 55°C and Figure 14 b at 70°C.

The data suggest that pretreatment generally enhances NH_4^+relative to untreated WAS.

Focusing on Figure 14 a, which examines NH_4^+ release at 55°C.



(a)



(b)

Figure 14 The ammonium release versus time observed after pretreatment (a) 55°C (b) 70°C with respect to the operation period of 16.5 cycles, n=2. Time 0 is the influent sample. The influent/untreated WAS was fed throughout the experiment semi-continuously**.**

It is evident that thermal pretreatment yields the highest ammonium concentrations.

Specifically, the average NH_4^+ concentrations between 0.8 and 3.8 days were as follows: thermal pretreatment (55°C): 22 ± 2 mg sCOD/gVS; thermal pretreatment with H_2O_2 (55°C): 11 ± 4 mg sCOD/gVS; thermal pretreatment with FeCl2 (55°C): 14 ± 1 mg sCOD/gVS; and thermal pretreatment with $FeCl_2$ and H_2O_2 (55°C): 10 ± 3 mg sCOD/gVS.

This pronounced release of NH_4^+ following thermal pretreatment aligns with established findings in the literature (Dauknys & Mažeikienė, 2023; Nazari et al., 2017). In contrast, the combination of thermal pretreatment with H_2O_2 results in a lower NH_4^+ release, which is also accompanied by the liberation of N_2 (see section 4.5). This phenomenon points towards the occurrence of nitrification and subsequent denitrification processes. To explore this hypothesis, a batch experiment was conducted wherein sludge was subjected to 55°C thermal treatment with the addition of H_2O_2. Samples were taken at different time intervals to measure NO_3 and NO_2 levels. The measurement revealed an initial surge in NO_3 concentrations, followed by a sharp decline and eventual stabilization as observed in Figure 15

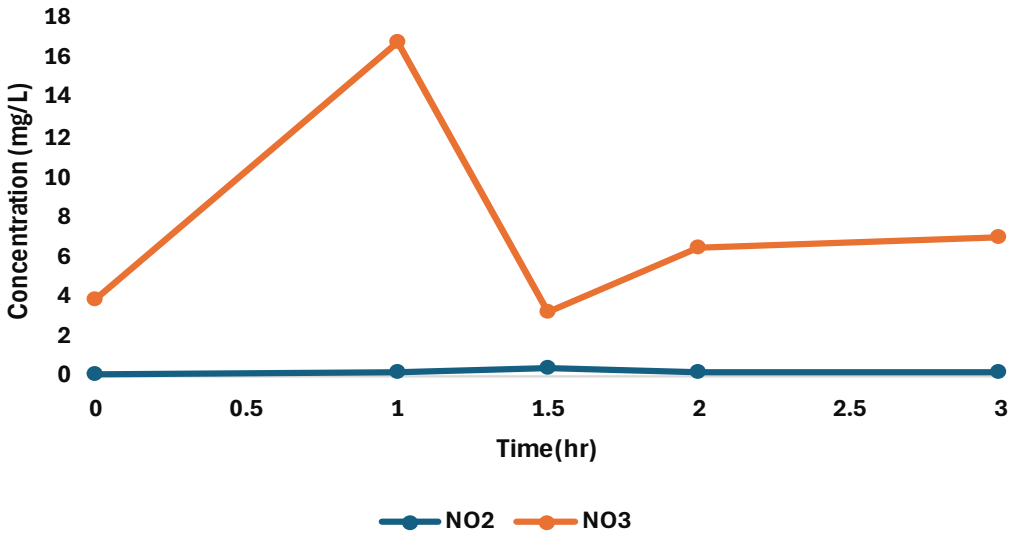


Figure 15 The release of NO_2^- and NO_3^- vs time at 55°C with dosing of 15mg/gTS of 30% w/w H_2O_2

The hypothesised mechanism behind this could be the following:

1. **Decomposition of H_2O_2**: H_2O_2 can be decomposed into H_2O and O_2 with the help of superoxidase, peroxidase which can lead to the production of trace O_2 (Sabumon, 2007).

$$H_2O_2 \rightarrow H_2O + O_2 \qquad \text{Equation 9}$$

2.**Nitrification**: $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$ Equation 10

3.**Denitrification**: $2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$ Equation 11

Where H_2O is water, H_2O_2 is hydrogen peroxide, NO_3^- is nitrate, NO_2^- is nitrite

The temperature of 55°C may have aided in the reaction to happen and give a competitive advantage to enrich the ammonium oxidising bacteria (AOB) in the possibility low DO concentrations(She et al., 2016).However, the NH_4^+ and N_2 production before and after in the batch test was not measured. Therefore, it is difficult to make a mass balance to further verify the hypothesised mechanism.

The mechanism underlying NH_4^+ release can be attributed to the hydrolysis of proteins into amino acids, which are subsequently converted into ammonium or fatty acids(Duong et al., 2019).The conversion of proteins to amino acids generally proceeds at a slower rate than the subsequent transformation to NH_4^+, rendering it the rate-limiting step in this process(Duong et al., 2019; Vavilin et al., 2008).Protease enzymes, which are pivotal in facilitating protein hydrolysis and peptide cleavage, mediate this conversion(Dary Guerra-Fajardo et al., 2022). However, the activity of these enzymes is highly sensitive to pH variations. Research has shown that in mildly acidic to neutral pH conditions, the degradation of proteins to NH_4^+ is inhibited, likely due to diminished protease activity. Breure et al. (1985) identified an optimal protease activity at a pH of 7.5, with a 50% reduction in activity observed at a pH of 5. A similar observation was reported by LU et al. (2007).This reduction may be attributed

to electrostatic repulsion among the charged active sites of the proteolytic enzymes under acidic conditions (Duong et al., 2019).

In this study, the pH levels recorded for the thermal with $FeCl_2$ and thermal with $FeCl_2$ and H_2O_2 treatments were around 5, which may account for the observed reduction in NH_4^+ release. This observation is consistent with the findings of Dauknys & Mažeikienė (2023), who reported a smaller increase in NH_4^+ release with iron-based oxides compared to treatments that did not incorporate Fe-based additives.

As depicted in Figure 14b, the range of NH_4^+ increase at 70°C closely follows the trend that was observed at 55°C. This similarity may be linked to reduced protease activity at elevated temperatures. Tang et al. (2010) observed that after incubation at 70°C, only 3% of the original protease activity remained, which corroborates the comparable levels of ammonium release at both temperatures despite the heightened thermal input.

4.4Effect of different pretreatment conditions on fermentative products and pH

VFAs and fermentative products are key intermediates in the anaerobic degradation of organic compounds during the acidogenic stage of fermentation. These VFAs, typically comprising fewer than six carbon atoms, include formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid (Althuri & Venkata Mohan, 2022). Among these, propanoic acid, acetic acid, and butyric acid are the most commonly produced and are of particular interest due to their significant role in biogas production (Vázquez-Fernández et al., 2022). In addition to VFAs, the current study also identified the presence of alcohols, primarily ethanol, which do not fall into the VFA category but are relevant to the discussion of fermentative products. Therefore, this section encompasses the analysis of both VFAs, and alcohols as measured in the study. Table 8 summarizes the presence of the major VFAs, and ethanol (in all the fermentative products) observed after the thermal and thermo-chemical pretreatments at (55°C) and pH observed after the pretreatment.

Table 8 The average fermentative products produced at 55°C, the major products reported over the operation period of 16.5 cycle the corresponding pH after pretreatment

Experiment	Total avg FP(mg/gVS)	% of acetic acid	% of butyric acid	% of propionic	% of ethanol	Initial pH	pH (55°C)
1	122	47	11	12	0	6.6	6.3
2	30	37	15	13	0	6.6	6.2
3	694	15	3	4	67	6.5	5
4	196	18	2	3	75	6.5	5.2

1: thermal,2: thermal with H_2O_2, 3: thermal with $FeCl_2$, 4: thermal with $FeCl_2$ and H_2O_2

The analysis focuses on the fermentative products generated at 55°C across different pretreatment methods. As illustrated in Figure 16, there is a noticeable increase in fermentative products production post-pretreatment, although this increase is not uniform across all methods.

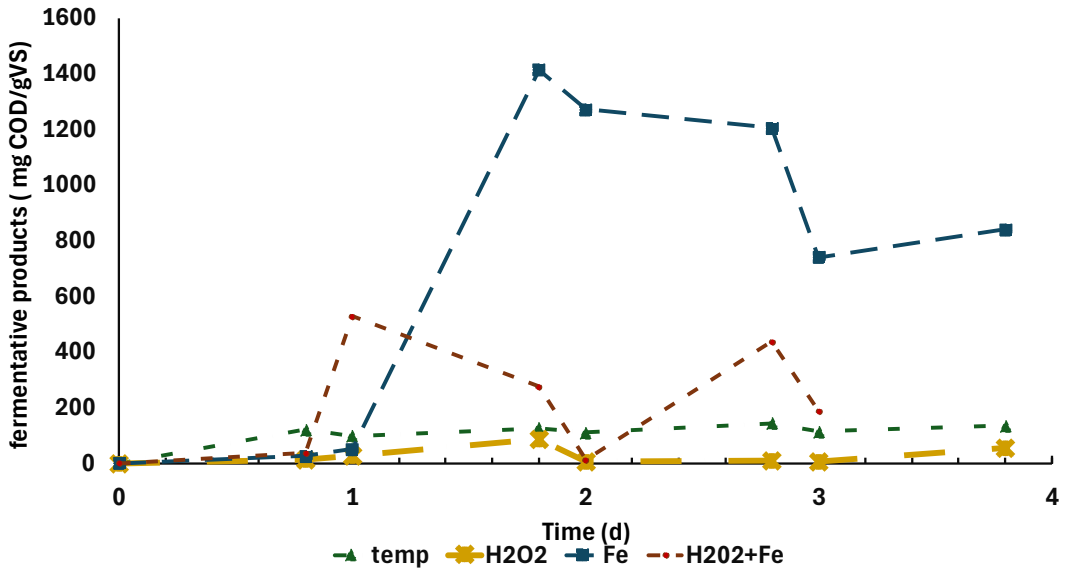


Figure 16 The fermentative products produced versus time for all pretreatment conditions at 55°C over operation period of 16.5 cycles.

Notably, the lowest fermentative product production was observed in the experiments involving thermal and H_2O_2 condition. This reduced production is likely attributable to the limited availability of convertible sCOD and the high oxidative potential of H_2O_2, which may inhibit the growth of fermentative bacteria necessary for converting organic material into fermentative products. Furthermore, a marked decrease in the formation of fermentative products was observed between days 2 and 3, largely due to the absence of butyric acid production during this period.

Conversely, the highest production of fermentative products was recorded following pretreatment with thermal and $FeCl_2$. This increase is primarily driven by ethanol production, which was uniquely observed in the experiments involving thermal and $FeCl_2$, as well as thermal $FeCl_2$ and H_2O_2 pretreatments. The emergence of ethanol suggests a shift in the fermentative pathway. The major fermentative pathways include the acetolactic, butyric, propanoic, and ethanol pathways, each leading to the production of acetic acid, butyric acid, propionic acid, and ethanol, respectively, with dominance varying by pathway (van Lier et al., 2020; Ren et al., 1997). The addition of Fe (II) appears to influence the fermentation process, directing it towards ethanol pathway. This shift is mechanistically linked to the activity of FeFe-H_2ases,an enzyme complex critical in ethanol fermentation by fermentative bacteria (Zheng et al., 2022) Research indicates that supplementation with Fe(II) upregulates genes encoding FeFe-H_2ases and associated enzymes, thereby facilitating the observed shift in fermentative outcomes (Z. Li et al., 2021; Yang & Wang, 2018; X. Zhao et al., 2017b).

4.5Effect of different pretreatment conditions on gas production

Biogas production represents the final stage of anaerobic digestion, wherein facultative anaerobic bacteria convert organic matter into various gases, including CH_4, H_2, hydrogen sulphide (H_2S), and carbon dioxide (CO_2) (El-kebeer et al., 2024). This study primarily focuses on the production of bio-H_2, examining gas production under various pretreatment conditions, particularly at 55°C. In the current study CO_2, H_2, and N_2, carbon monoxide (CO) and CH_4 were measured. It is important to note that H_2S was not measured in this study due to the unavailability of the necessary apparatus.

Figure 17 presents the cumulative gas production observed across different pretreatment experiments at the off gas of 55°C reactor over 16.5 cycles.

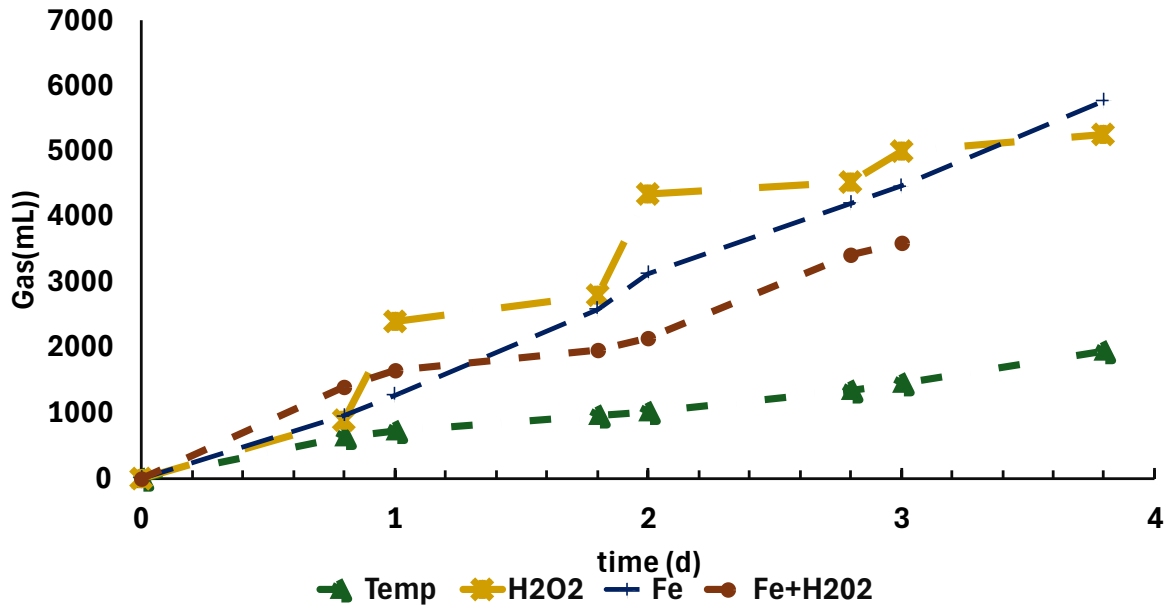


Figure 17 The cumulative gas production vs time observed at the off gas of 55 °C reactor over 16.5 cycles (with N_2)

Notably, gas production was detected only at 55°C, with no measurable gas production at 70°C. This absence of biogas production at the higher temperature may be attributed to prolonged exposure to 70°C, likely creating an environment unfavourable for biogas generation.

Under each pretreatment condition at 55°C, the production of CO_2, H_2, and N_2 was observed, with CO also detected in the thermal with H_2O_2 and thermal with $FeCl_2$ and H_2O_2 pretreatments. In the thermal with H_2O_2 pretreatment, the generation of N_2 can be linked to nitrification-denitrification, as discussed in (Section 4.3). Among the various conditions, thermal with $FeCl_2$ exhibited a higher steady gas production. In contrast, the gas production pattern in the thermal with H_2O_2 condition displayed a saw-tooth pattern, possibly due to fluctuations in CO and N_2 levels, although the exact mechanism underlying this trend remains unclear.

Figure 18 depicts the percentage of total sludge COD converted to gas COD, excluding N_2 content. For the thermal pretreatment, thermal pretreatment with $FeCl_2$, and H_2O_2 conditions, less than 0.05% of COD was converted to gas COD.

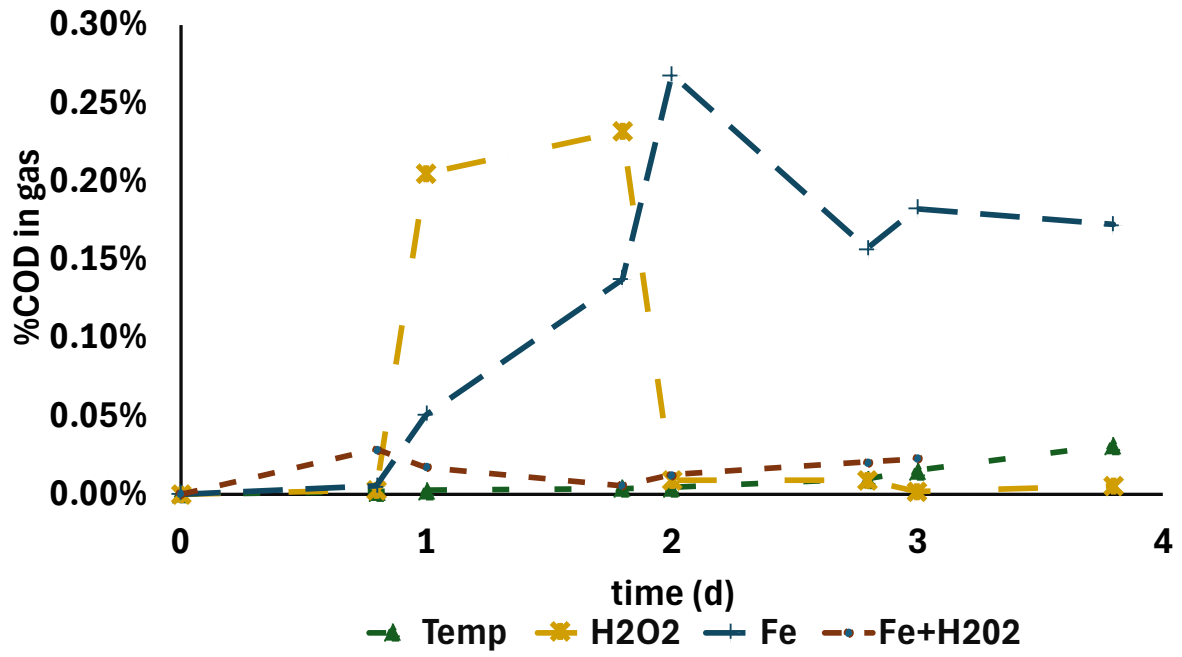


Figure 18 The percentage of total sludge COD in gas vs time for all pretreatment conditions over an operation period of 16.5 cycles

CH_4 production was observed to commence after day 2 in the thermal pretreatment, indicating a low but a steady increasing trend which is profound after day 3. In the thermal with H_2O_2 condition, peaks in gas production were recorded at day 0.8 and day 1.8, likely associated with CH_4 generation, which was just observed on day 0.8 and day1.8. This can be likely due to improper mixing leading to some pockets of sludge having higher SRT, promoting the growth of methanogens in those pockets. The

highest conversion percentage, approximately 0.25%, was observed in the thermal pretreatment with $FeCl_2$.

Figure 19 shows the proportion of H_2 COD relative to total COD (H_2 COD/tCOD) in the sludge after pretreatment at 55°C.

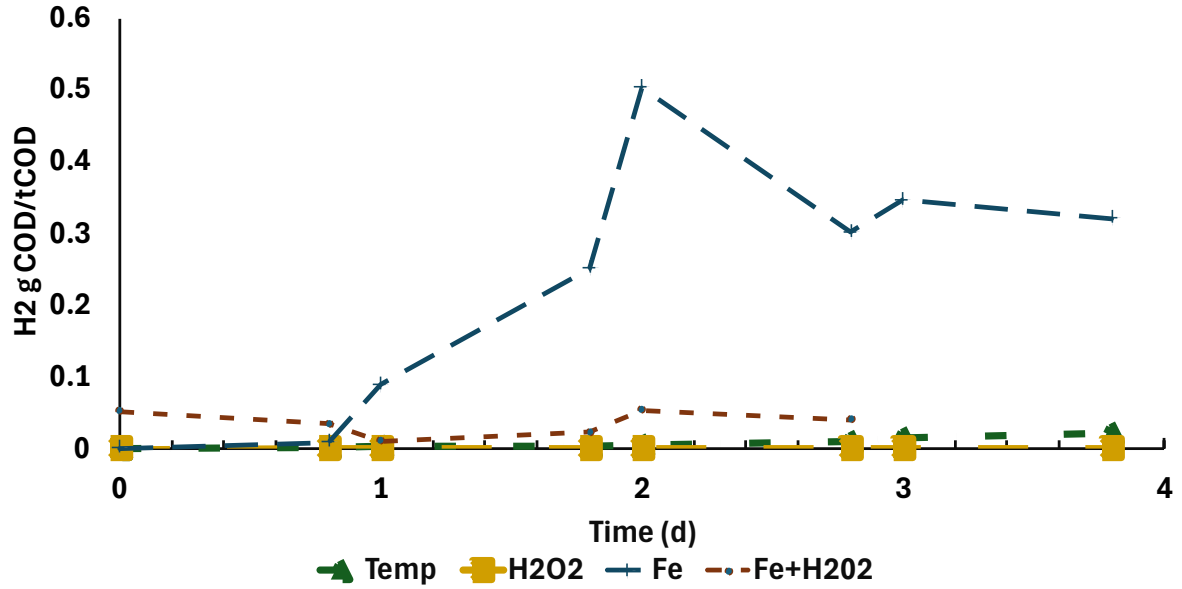


Figure 19 The H_2 COD in total sludge COD versus time for all the pretreatment conditions over the operation period of 16.5 cycle

In the thermal and thermal with H_2O_2 conditions, negligible H_2 COD/tCOD was detected. The highest H_2 COD /tCOD was observed in the thermal with $FeCl_2$ condition, which is likely due to the role of iron in enhancing ethanol fermentation. This observation aligns with the peaks in ethanol and H_2 production observed around day 2.

From Figures 18 and 19, it can be inferred that the overall conversion percentage of sludge COD to gas COD or H_2 COD remains low across all pretreatment conditions (less than 0.5% and 0.6 respectively). This low conversion efficiency may be attributed to the activity of HCB, which utilize H_2 for various metabolic processes. The primary consumers of hydrogen in anaerobic digestion include methanogens, SRB, and homoacetogens, the latter of which produce acetate(van Lier et al., 2020). Theoretically, Homoacetogens typically require 4 moles of H_2 to synthesize 1 mole of acetate (Y. Zhao et al., 2010). As given by equation 12

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O \qquad \text{Equation 12}$$

Given the pH of 5 observed in the thermal with $FeCl_2$ condition, the presence of methanogens and SRBs is unlikely, suggesting that homoacetogens may be the dominant HCB in this scenario. This observation underscores the significant influence of pH on gas production.

Suppressing methanogens is achievable through various strategies, including operating at shorter SRTs, as employed in this study. However, the suppression of homoacetogens presents a greater challenge. Some homoacetogens, such as *Clostridium ljungdahlii, Clostridium autotrophicum*, and *Clostridium aceticum*, possess the ability to form spores, enabling their survival through pretreatment processes (Wan et al., 2016b).

The literature consistently reports low conversion percentage of sludge COD to H_2 COD under mildly acidic to neutral pH conditions (El-Qelish et al., 2020; Wan et al., 2016b; Y. Zhao et al., 2010). For instance, conversion percentage from glucose COD to H_2 COD range from 4% to 8.2% when heat-pretreated sludge is used as the hydrogen producer, with glucose as the substrate (Wan et al., 2016b). Considering that WAS is a far more complex substrate than glucose for H_2 production, achieving higher conversion rates remains a substantial challenge.

CH_4 production was observed with the thermal pretreatment condition. Despite the SRT being designed to suppress methanogenic activity, the presence of CH_4 can be explained by the combined effects of temperature, pH, and the growth kinetics of hydrogenotrophic methanogens. These methanogens, characterized by a shorter doubling time (td is 0.7 days), exhibit higher growth rates compared to acetoclastic methanogens (td is 5.8 days) (van Lier et al., 2020). The pH of 6.3 observed in the thermal pretreatment further facilitated methanogen activity. In contrast, CH_4 was detected only briefly in the thermal with H_2O_2 treatment. Despite a pH of 6.2, no sustained CH_4 production occurred, potentially due to CO production, which is known to inhibit methanogenic activity (Schöne & Rother, 2018).

The low yields of H_2 and CH_4 in the thermal with H_2O_2 treatment may be attributable to changes in oxidation-reduction potential (ORP) and the high oxidative power of H_2O_2, which likely inhibited the microbial growth necessary for sustained gas production.

4.6 Role of pH in changing fermentative pathways with Fe (II) addition

The results from the fermentative products and gas production(H_2) analyses suggest a shift in metabolic pathways, potentially driven by the addition of iron. This shift could be attributed to the enhancement effects of Fe (II) or the acidic pH conditions, which may have suppressed methanogenic activity. To explore this further, two parallel semi-continuous tests of thermal pretreatment at 55°C were conducted. In the first test, $FeCl_2$ was added at a dosage of 15 mg/gTS, with the pH adjusted to 6 using 2M NaOH. In the second test, no $FeCl_2$ was added, and the pH was lowered to 5, mimicking the final pH observed in the thermal with $FeCl_2$ treatment, by adding HCl.

Figure 20 provides an overview of gas production and fermentative products during the experimental period. It specifically details the fermentative products, while Figure 21 illustrates the percentage of sludge COD converted to gas, and Figure 22 shows H_2 production in milliliters. The figures depict a comparison of four experimental conditions: thermal pretreatment at 55°C (temp), thermal pretreatment with $FeCl_2$ at pH 5 (Fe, pH=5), thermal pretreatment with $FeCl_2$ and NaOH adjustment to pH 6 (Fe +

NaOH), and thermal pretreatment with HCl adjustment to pH 5 (HCl). This experimental run was conducted over three days, 0.8 days shorter than the other pretreatment conditions. The solubilization patterns of sCOD and NH_4 are provided in Annexure 2.

In Figure 20, a clear trend in fermentative product production is observed.

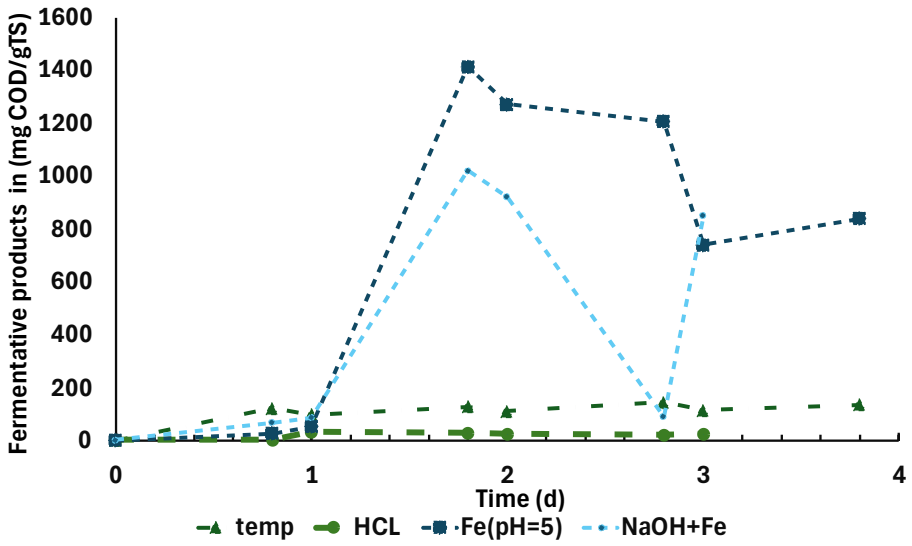


Figure 20 The fermentative products vs time formed in the after thermal with $FeCl_2$ and NaOH and thermal with HCl (55°C) compared with the thermal and thermal with $FeCl_2$ conditions(55°C)

The thermal pretreatment with $FeCl_2$ and NaOH condition reported ethanol production similar to the thermal pretreatment with $FeCl_2$, with a significant increase noted at 1.8 days. However, an anomaly was observed on the third day, where ethanol production decreased unexpectedly. In contrast, the thermal with HCl condition resulted in the production of VFAs without any ethanol production. Figure 21 depicts the percentage of sludge COD converted to gas.

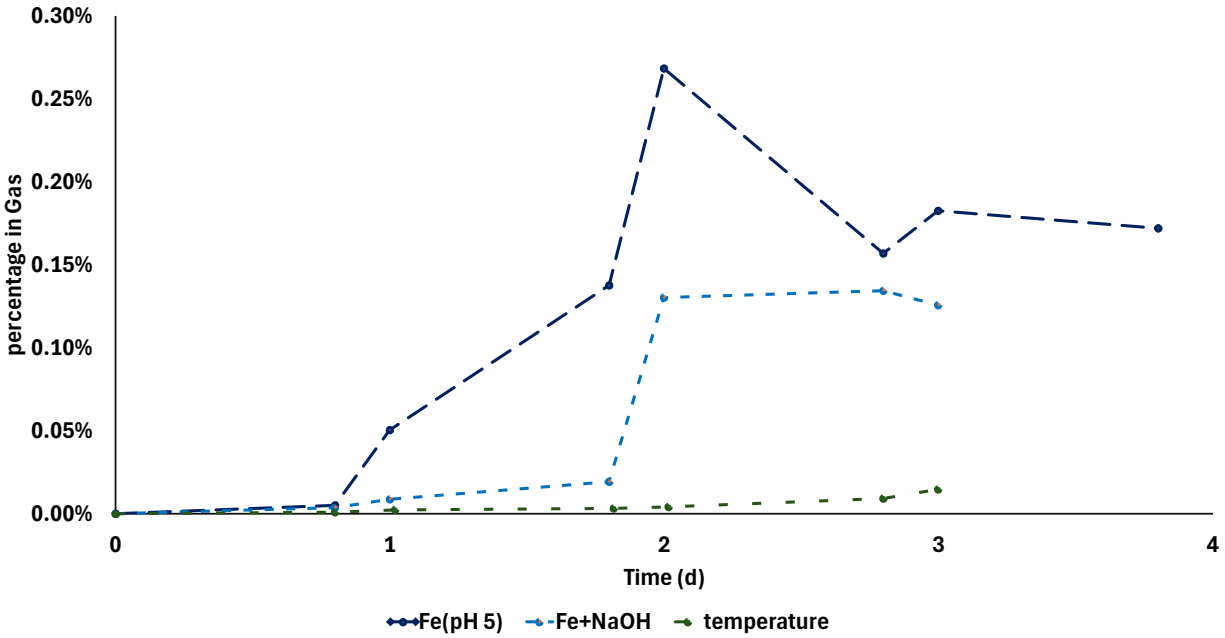


Figure 21 The percentage sludge COD in gas formed after thermal with $FeCl_2$ and NaOH and thermal with HCl (55°C) compared with the thermal and thermal with $FeCl_2$ conditions(55°C)

Notably, no gas production was observed under the thermal pretreatment with HCl condition. The conversion percentage of sludge COD to gas was higher in the thermal pretreatment with $FeCl_2$ at pH 6 compared to thermal pretreatment alone.CH_4 production was also observed, contributing to the increased gas COD on days 2 and 2.8.

In Figure 22, it is evident that the thermal pretreatment with $FeCl_2$ at pH 6 produced less H_2 compared to the thermal pretreatment with $FeCl_2$ alone.

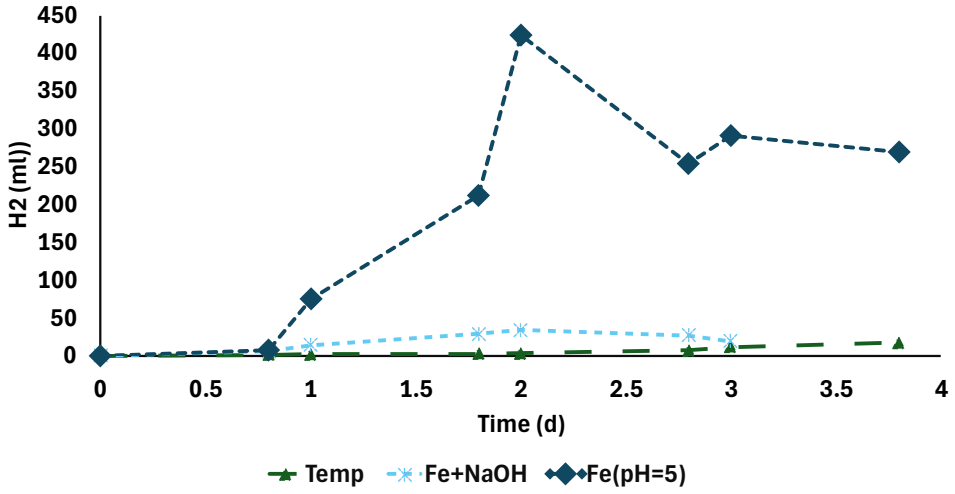


Figure 22 Cumulative H_2 produced formed after thermal with $FeCl_2$ and NaOH and thermal with HCl (55°C) compared with the thermal and thermal with $FeCl_2$ conditions(55°C).

This discrepancy is likely due to the pH effect, where the higher pH favoured the growth of methanogens, which could consume H_2. Additionally, the sludge pretreated with thermal and $FeCl_2$ at pH 6 was visually darker and emitted an H_2S-like odour, suggesting the possible presence of SRB that might compete for H_2.

Therefore, this study highlights there seems to exist complex or a synergetic effect between iron addition, pH and the resulting metabolic pathways during anaerobic digestion. $FeCl_2$ combined with pH adjustments can significantly influence the balance between H_2 and CH_4 production, as well as the type of fermentative products formed.

4.7 Effect of different pretreatment conditions on Biomethane Potential(BMP)

BMP provides critical insights into the biodegradability of substrates and their potential for methane production through AD processes (Steven T Sell et al., 2010). By allowing for a direct assessment of biogas yields, BMP serves as an essential metric in optimizing AD operations (Jingura & Kamusoko, 2017). In this study, BMP was calculated using the methodology outlined in Equation 8 of section 3.5. Figure 23 illustrates the BMP results for untreated WAS and pretreated sludge subjected to 70°C under various pretreatment conditions. Despite the setup including both a 55°C and a 70°C reactor, the sludge fed to the anaerobic digester—and subsequently used for the BMP analysis—was taken from the 70°C reactor. This reflects the process in full-scale operation, where the pretreated sludge from the 70°C reactor will be fed into the anaerobic digester following the Themista® system

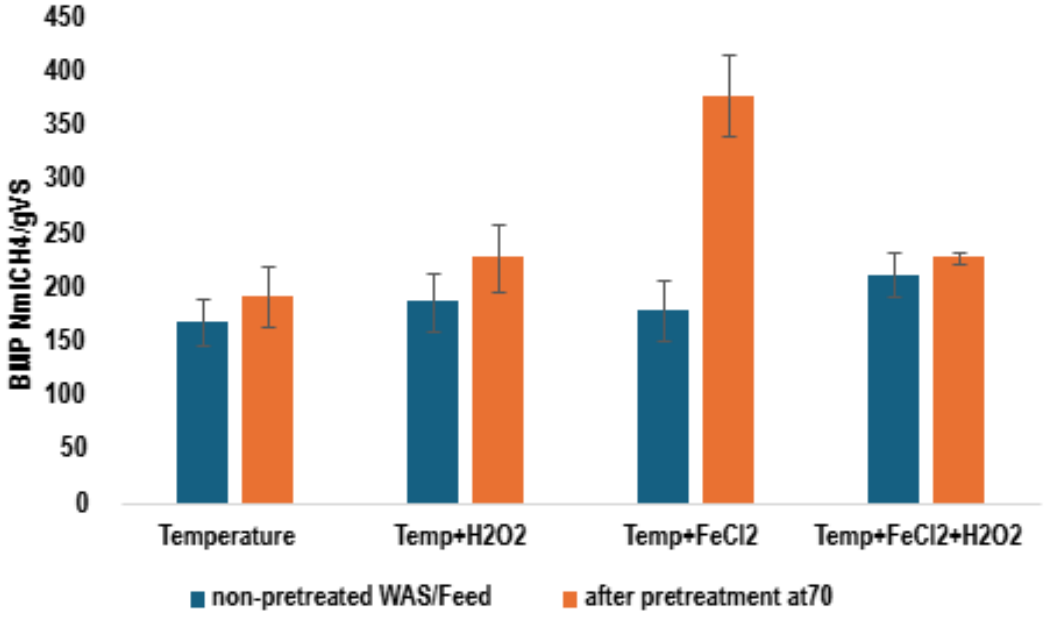


Figure 23 The BMP of the untreated WAS and pretreatment after 70°C for all pretreatment conditions

The data indicate that, across the pretreatment conditions involving only thermal, thermal with H_2O_2, and thermal with $FeCl_2$ and H_2O_2. Thise increase in BMP from the untreated WAS to the pretreated

sludge was not significant ($p<0.05$). This observation is consistent with findings reported by Nazari et al. (2017d) and Gonzalez (2022b), who noted similar outcomes following pretreatment with temperature and temperature combined with H_2O_2, respectively.

It should be noted that the BMP results exhibit a high standard deviation, which suggests that the observed increase between the untreated WAS and pretreatment samples may be insignificant. This variability could be attributed to potential issues such as improper mixing and inadequate mass transfer during the BMP assays, likely due to limitations in the mixing equipment. These factors may have led to precise but inaccurate results among the triplicate samples, an issue also reported by Gonzalez (2022b).

However, a marked increase in BMP was observed following pretreatment with thermal and $FeCl_2$. In thermophilic digestion systems, syntrophic bacteria play a crucial role in converting acetate into CO_2 and H_2, thereby promoting hydrogenotrophic methanogenesis. The addition of Fe can significantly enhance the activity of key enzymes in this pathway, accelerating acetate degradation and facilitating higher methane yields while concurrently reducing volatile fatty acid (VFA) concentrations (Chen et al., 2023). Although literature specifically addressing BMP post-$FeCl_2$ pretreatment is limited, existing studies on anaerobic digestion with Fe supplementation consistently report enhanced CH_4 yields compared to systems without Fe addition. Chen et al. (2023), for instance, investigated the effects of Fe(II) and carbon addition in the anaerobic digestion of kitchen waste at 55°C, documenting a significant increase in cumulative methane yield in reactors supplemented with Fe.

A plausible explanation, particularly for the lower increase in BMP in the thermal with H_2O_2 is the decomposition of H_2O_2 into H_2O and O_2, which could temporarily introduce micro-aeration type conditions within the system. If the oxygen exposure is sufficiently high, facultative organisms may outcompete strict anaerobes over time due to their higher specific activities and growth rates. This competition for substrates could inhibit methanogen activity, ultimately leading to reduced methane production, as suggested by Botheju (2011).

Furthermore, the percentage of initial COD converted to CH_4 was calculated (with detailed calculations provided in Annexure 3). The conversion percentage were as follows: thermal pretreatment: 38%; thermal pretreatment with H_2O_2: 46%; thermal pretreatment with $FeCl_2$: 76%; and thermal pretreatment with $FeCl_2$ and H_2O_2: 46%. According to Metcalf & Eddy (2003), a well-optimized anaerobic digestion setup should exhibit a COD conversion percentage between 50% and 70%. It can be observed that the COD conversion percentage for thermal pretreatment, thermal pretreatment with H_2O_2, and thermal pretreatment with H_2O_2 and $FeCl_2$ fall below 50%. This lower conversion percentage may be attributed to the prolonged exposure to thermal and thermo-oxidative processes, which could potentially transform soluble organics into more refractory compounds that are slower to biodegrade, as reported by Özön & Erdinçler(2019),Eskicioglu et al. (2008),Shahriari et al. (2012)with pretreatment of microwave and H_2O_2 at a temperature between 60-80°C.

5.Overall Discussions

5.1 Ethanol Fermentation

The observation that ethanol constitutes over 50% of the fermentative products in the thermal (55°C) $FeCl_2$ condition (section 4.4) indicates a significant shift towards ethanol fermentation. This pathway is known for its high hydrogen production efficiency compared to other fermentation routes(Ren et al., 1997). This aligns with the results of this study. Theoretical stoichiometry suggests that ethanol can produce 2 moles of H_2 per mole of ethanol (Y. Zhao et al., 2010; Zheng et al., 2022). The equation for ethanol fermentation is given by equation 13

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad \text{Equation 13}$$

The ethanol fermentation pathway is facilitated by hydrogenase enzymes, particularly FeFe-H_2ases, which are more effective in hydrogen production than NiFe-H_2ases. The Fe-S clusters in these hydrogenases serve as sites for hydrogen oxidation and evolution, and they also facilitate electron transfer between external electron carriers (e.g., NADH) and the hydrogen cluster (Yang & Wang, 2018; X. Zhao et al., 2017b). These Fe-S clusters, known as ferredoxins, are crucial for efficient electron transport and hydrogen production.

The type of HPB and the fermentative pathways used by the microbial community are detailed in section 2.6.2 and section 2.6.3. Literature suggests that the key microbial community facilitating ethanol-type fermentation includes *Ethanoligenens* and *Ethanoligenens harbinense*. The primary end-products of *Ethanoligenens* are ethanol, acetic acid, H_2, and CO_2, which may serve as metabolic intermediates for other microbial partners, such as methanogens, acetogens, iron and sulphate reducers, and denitrifiers. These bacteria are acid-tolerant and have significant potential for syntrophic interactions with methanogens due to their H_2-ethanol co-production characteristics.

In the current study, as discussed in section 4.4, the presence of all the key end-products produced by *Ethanoligenens* and *Ethanoligenens harbinense* suggests the potential presence of these bacteria. The higher BMP observed in the thermal pretreatment with $FeCl_2$, along with a higher COD-to-CH_4 conversion percentage, further supports this hypothesis. These bacteria contain genes encoding ferredoxins and enzymes such as alcohol dehydrogenase (Adh) and acetate kinase (Ack), which are integral to the ethanol-acetic acid fermentation pathway (Z. Li et al., 2021). The addition of Fe can enhance the availability of these essential metabolites and promote the growth of related bacterial communities, as corroborated by previous studies (S. Wang et al., 2020; Yang & Wang, 2018; X. Zhao et al., 2017b). This can further explain the higher H_2 production that was observed due to the addition of $FeCl_2$ in this study.

pH plays an important role in determining the type of gas that can produced, and this can be observed from the current study. Ethanol pathway has been reported be observed between the pH of 4 to 5.3(section 2.6.4). In this study the higher yield of H_2 was observed at the pH of 5 However from section 4.6 it can also be inferred that the addition of $FeCl_2$ and a pH of around 5 only leads to an enhancement

of H_2 yield. Which further indicated towards the synergetic effect of pH and $FeCl_2$ addition. The results from section 4.6 also indicate that even at a pH of 6, the ethanol pathway can be observed. There are fewer studies which have indicated that. Fe Addition at a higher pH reported lower yields of H_2 production as simultaneously CH_4 production was observed. This indicated towards a very complex dynamics of pathway, dictated strictly by pH and facilitated by specific organics which are pH sensitive

5.2The role of $FeCl_2$ and H_2O_2 Fenton reaction

Fenton reaction is typically the chain reaction between Fe and H_2O_2. The reaction produces hydroxyl radicals which oxidize the target compounds. The vital parameters for optimum Fenton type of condition to happen are the dosage of H_2O_2, pH effect, temperature and the type of Fe used. Even though Fe (III) can be used however, research suggest that addition of Fe(II) is better for catalysing the reactions (Neyens et al., 2003). Gonzalez (2022b) hypothesised that there might be a Fenton reaction happening which enhanced the biodegradation that was observed in the study after pre-treating WAS at 70°C with H_2O_2. To further understand the process, in this study, the addition of $FeCl_2$ and H_2O_2 with thermal pretreatment was conducted.

The results of the current study provide a very conducive environment for Fenton to happen. As addition of Fe and H_2O_2 is being added at 55°C and as temperature rises above 40°C the rate of reaction increases as well. However, the sCOD release and solubilisation patterns are similar to the thermal and the other thermo-chemical pretreatments reported in this study. There can be multiple reasons for it. The final pH reported in our study was 5. However, Fenton occurs in more acidic pH of around 3(Pilli et al., 2015b). Secondly, the dosage of H_2O_2 plays a vital role. In our study the dosage of H_2O_2 was equal to the dosage of Fe(II) addition that is 15mg/gTS. Pham et al., (2011); Ramaswamy et al., (2009)have reported a dosage of 1 part of Fe per 5-25 parts of H_2O_2. Zhou et al. (2015) have reported a Fe dosage of 16.3mgFe/gTS and H_2O_2 concentration of 28 to 30mg/gTS for optimum dewatering of sludge. (Pilli et al., 2015a) reported that if required $H_2O_2$is not added, the targeted reduction in COD cannot be achieved. A combination of these factors can be a plausible reason why a Fenton type reaction was not effectively observed.

5.3 Estimation of H_2 production in the full-scale

This section seeks to estimate how the results of this study could be applied to the full-scale operation of the Themista® system. The primary focus is on evaluating the potential for H_2 production if the thermal and thermo-chemical pretreatments at 55°C, as tested in this study, were implemented on a larger scale. To estimate the potential H_2 output, the average percentage of H_2 COD in total sludge COD from each experiment was used as a key metric. This value, combined with the daily COD load of the full-scale Themista® system, provided a basis for calculating potential H_2 production. In the full-scale Themista® system, the primary aim of H_2 production is to enhance biomethanation by injecting the generated H_2 into the anaerobic digesters. This section's objective is to offer a preliminary estimate of

potential H_2 and CH_4production based on experimental data, evaluated under two different scenarios. Given the preliminary nature of these estimates, the calculations should be interpreted with caution. These calculations were performed in accordance with parameters provided by the stakeholders of the Themista project, with detailed methodologies outlined in the annexure 4.

It is essential to note that the full-scale Themista system consists of three 55°C reactor tanks in the initial stage of pretreatment. The calculations presented here take into account the operation of these three reactors under the following two scenarios:

1. **H_2 Production as Fuel**: Estimating the number of kilometers that could be covered using the H_2 produced. (considering 1kg H_2 can be s fuel for 100kms of driving)
2. **CH_4 Production**: Estimating the potential increase in CH_4 production after injecting the H_2 produced in the Themista system at 55°C into the anaerobic digesters.

To estimate the enhancement of biomethanation from the H_2 produced at 55°C, Equation 14 was employed. The calculations are based on several assumptions: hydrogenotrophic methanogenesis occurs within the anaerobic digesters, mass transfer is optimal, and mixing within the digesters is perfect. Additionally, it is assumed that the Themista system operates with similar efficiency as the lab-scale setup.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{Equation 14}$$

Table 9 The overview of the estimated H_2 produced at Themista® and the estimated methane that production at anaerobic digestor.

Experiment	H_2 produced (kg/day)	Calorific value of H_2 produced (MJ/day)	Kms that can be covered per day	Contribution of CH_4production at digestor
Thermal	1.6	204	170	0.1%
Thermal with H_2O_2	1.7	215	179	0.1 %
Thermal with $FeCl_2$	17.9	2147	1789	1.0%
Thermal with H_2O_2 and $FeCl_2$	3.0	365	304	0.2%

Preliminary calculations suggest a positive commercial potential for utilizing the produced H_2 as fuel, indicating that H_2 could have viable applications in a commercial context. However, while these calculations show that the production of CH_4 production following the addition of H_2 over the current CH_4 production contributed by the addition of H_2 in the digester is less than 1% across all pretreatment conditions, it remains challenging to accurately estimate the true extent of CH_4 enhancement in

anaerobic digesters due to the introduction of H_2. The simplifications inherent in these calculations, along with idealized conditions, may lead to an overestimation of CH_4 production, and the actual increase could be even lower in real-world applications. This is further complicated by the specific characteristics of the sewage sludge at Kralingseveer, mixing efficiency of Themista® etc which could significantly influence the outcomes.

Section 2 of the theoretical background provides an overview of H_2 production from sewage sludge and discusses relevant literature. However, it is crucial to acknowledge the significant challenges associated with H_2 production from sewage sludge, particularly the typically low yields observed, as highlighted in this study. Even the theoretical maximum yield of bio-H_2, which is 4 moles of H_2 per mole of glucose (equation 4), can only be achieved under conditions where the sole electron sinks are H_2 and acetate. This theoretical maximum represents only a 25% conversion of substrate electron equivalents into bio-H_2(Lee et al., 2010). In practice, however, other organic sinks—such as butyrate, propionate, ethanol, lactate, and biomass—are generated in significant quantities, further reducing the maximum yield.

The heterogeneity and composition of sewage sludge present additional challenges for H_2 production. For instance, the presence of heavy metals such as Hg, Cu can inhibit H_2 production(Ananthi et al., 2024b). Studies have shown that carbohydrate-rich substrates can produce up to 20 times more H_2 than protein- and lipid-rich substrates(Yao et al., 2018a). Additionally, the carbon-to-nitrogen (C/N) ratio is critical for optimizing H_2 production. An optimal C/N ratio of 47 has been reported Yao et al. (2018a) and Sreela-or et al. (2011)whereas sewage sludge typically has a C/N ratio of 4-10 (Yang & Wang, 2019). The C/N ratio was not measured in this study, which could have implications for the observed H_2 production.

As discussed in Section 2.6, dark fermentation is considered a relatively economical and sustainable method for H_2 production compared to other methods. However, its low yield is a significant limitation, as H_2 is often consumed by methanogens or SRB before it can be harvested. This necessitates careful adjustment of process parameters such as pH and HRT. The current study indicates that even with adjusted HRT, if the pH remains around 6, methanogens or hypothesized SRBs may proliferate. Lower pH only with Fe dosing, improved H_2 production compared to other experiments. However, the consistency of this improvement remains uncertain, and the need for chemical additions raises questions about the sustainability of this approach. For full-scale application, a cost analysis of Fe addition is essential to determine whether H_2 production is both economically viable and sustainable.

6.General conclusions

This study evaluated hydrolysis and bio-H_2 production LTTP and LTTP with chemical additives at 55°C, modeled after the Themista system's full-scale setup. The experiments demonstrated that all pretreatment methods led to increased sCOD release compared to untreated WAS. The average release over the 16.5 cycle operation cycle were, thermal : 249 ± 9 mg/gVS , thermal with H_2O_2 : 236 ± 9 mg/gVS to, thermal with $FeCl_2$: 245 ± 13 mg/gVS, thermal with $FeCl_2$ with H_2O_2 : 253 ± 22 mg/gVS. This increase in solubilization was confirmed by EPS results, which showed an enhanced s-EPS fraction of proteins, humic substances, and carbohydrates, alongside a reduction in the TB-EPS fraction post-treatment.

Gas production was only observed at 55°C, with the highest bio-H_2 yield occurring under thermal pretreatment with $FeCl_2$. The presence of Fe at a pH of 5 facilitated a shift towards ethanol fermentation, further supporting bio-H_2 production. The BMP analysis revealed that while all pretreatment conditions except thermal with $FeCl_2$ have less than 50% conversion of initial COD to methane, the addition of $FeCl_2$ substantially increased the BMP, achieving a conversion percentage of 75%, indicating its potential to enhance both bio-H_2 and methane production.

This thesis sheds light on the underreported potential of gas production during pretreatment stages, highlighting the significant roles of pH, HRT, and temperature and their interplay on H_2 production. The findings suggest that thermal pretreatment, particularly with $FeCl_2$, holds a higher commercial value for bio-H_2 production, demonstrating that Fe acts as a biological enhancer rather than merely a chemical additive.

6.1 Answer to the Research Questions

1. **How does a two-phased thermal pretreatment at 55°C impact the efficiency of sludge hydrolysis and the quality and quantity of gas production?**

Sludge Hydrolysis: The efficiency of sludge hydrolysis is improved, as evidenced by increased sludge solubilization, with sCOD from 32 ± 9 mg/gVS to 249 ± 9 mg/gVS within 0.8days. The net increase in soluble protein was 4.9± 2.1 mg/gVS, and NH_4 increased from 3 ± 1 to 21 ± 1 mg/gVS within 0.8 days, maintaining a stable average increase of 22 ± 1 mg/gVS over the experimental duration.

Gas Production: The off-gas at 55°C contained a mixture of N_2, CH_4, CO_2, and H_2. The average percentage conversion of sludge COD to gas COD was less than 0.01%, with a similar conversion percentage for H_2/tCOD. A slow but steady increase in CH_4 production was observed between days 1 and 3.8.

2. **How does a two-phased thermal pretreatment at 55°C with the addition of $FeCl_2$ and H_2O_2 impact the efficiency of sludge hydrolysis and the quality and quantity of gas production?**

Sludge Hydrolysis: The thermo-chemical pretreatment significantly enhanced sludge solubilization, with sCOD release in the thermal with H_2O_2 condition increasing from 32 ± 9 mg/gVS to 249 ± 9 mg/gVS within 0.8 days. In the thermal with $FeCl_2$ condition, sCOD release increased from 11 ± 13

mg/gVS to 234 ± 13 mg/gVS within 0.8 days. For the thermal with H_2O_2 and $FeCl_2$ condition, sCOD release increased from 27 ± 22 mg/gVS to 271 ± 22 mg/gVS within 0.8 days.

Gas Production: The off-gas at 55°C primarily contained N_2, H_2, and CO_2, with CO production observed in the thermal with H_2O_2 and thermal with both H_2O_2 and $FeCl_2$ conditions. N_2 production was noted in the thermal with H_2O_2 condition, likely due to nitrification and denitrification. The maximum H_2 production was observed with thermal pretreatment and $FeCl_2$ addition, with an average sludge COD to gas COD conversion of around 0.1% and a similar H_2/tCOD conversion. Ethanol was detected in the fermentative products, indicating a metabolic shift to the ethanol pathway. H_2 production was pH-dependent, with higher yields at pH 5. Other thermo-chemical pretreatments showed an average sludge COD to gas COD conversion of less than 0.06%, with a similar H_2/tCOD conversion percentage. Figure 24 summarises the overall conclusions in terms of solubilisation and the possible fermentative pathways for each pretreatment condition reported in this study.

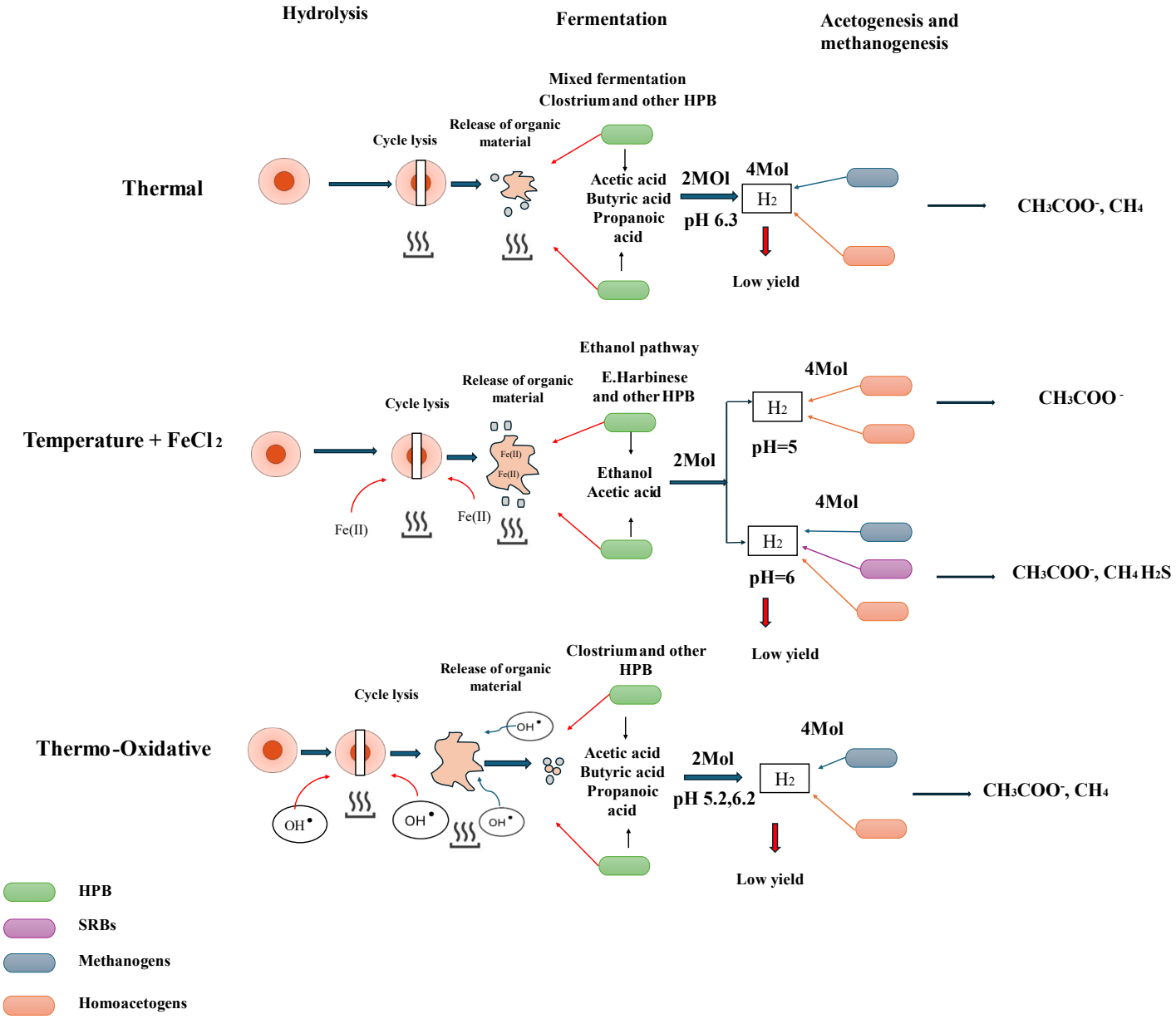


Figure 24 The overall conclusion of this study for various pretreatment conditions summarised with the biological pathways likely observed in this study

7. Future outlooks and recommendations

This study provides valuable insights for both the research community and practitioners regarding gas production during the pretreatment stage and the various interactions between factors that influence it. Additionally, it highlights the potential of Fe(II) as an enhancer of H_2 production and emphasizes the role of Fe as a biological enhancer in the process. These findings can guide the research community to further explore the potential of Fe(II) in H_2 production and encourage practitioners to consider implementing it on a larger scale.

Research Focus

This study underscores the significant role that Fe (II) can play in enhancing bio-H_2 production. Future research could investigate different concentrations of Fe (II) dosing and their impact on H_2 production, alongside measuring the activity of hydrogenase enzymes. Further exploration of alternative sources of Fe, such as scrap iron or Fe-rich sludge, could be conducted to assess their impact on H_2 production, thereby contributing to the sustainability of the process. Additionally, studies aimed at optimizing the doses of $FeCl_2$ and H_2O_2 could be undertaken to better understand their effects on H_2 production.

Themista® Process

An economic study is essential to evaluate the feasibility of H_2 production through Fe addition. Based on these findings, a decision should be made regarding the utilization of H_2, whether as a commercial fuel or for enhancing CH_4 production in digesters. This decision will also inform whether efforts should focus on enhancing H_2 production or reducing it.

1. **Enhancing hydrogen:** The Themista® system could be remodeled as an H_2 fermentation plus LTTP setup, rather than a two-step thermal pretreatment. In this scenario, the 55°C reactor could serve as the H_2 fermenter. Alternatively, the enhancement of HPB cultures could be achieved through the use of commercial enzymes or by isolating HPB strains. Furthermore, the application of Themista can be extended to the treatment of industrial sludge
2. **Reduction in H_2 production**: To reduce H_2 production, the SRT could be set between 6-10hrs, and the pH can be increased to 6.5.

Annexure 1

COD for Protein: 1.5mg COD/ mg protein

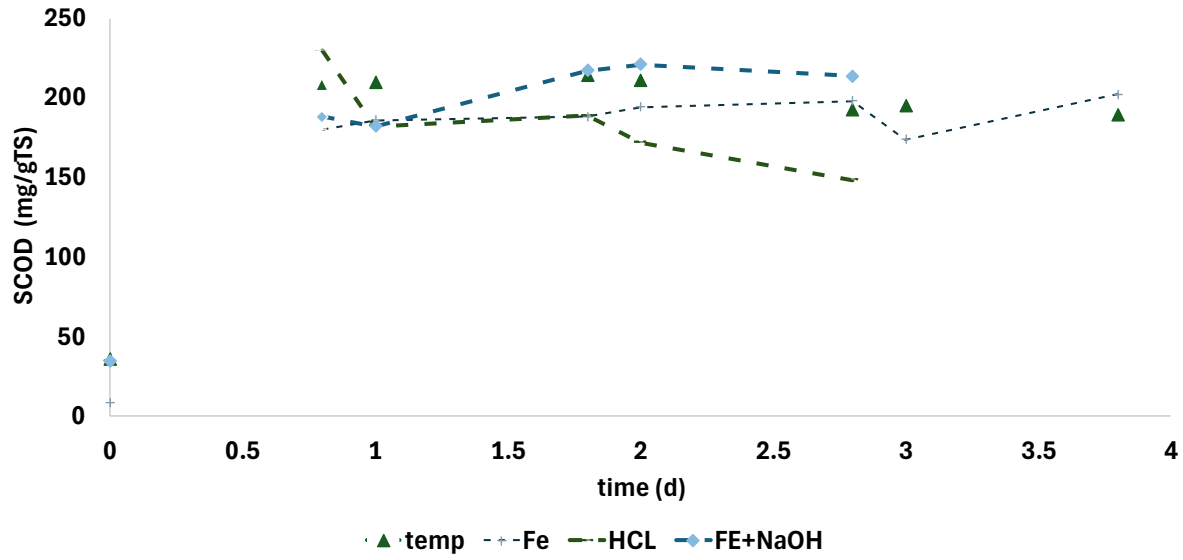
COD of Carbohydrates = 1.07mgCOD/L

		Protein(mg/gVS)	humics(mg/gVS)	carbohydrates(mg/gVS)	TCarbs(mg/gVS)	total P (mg/gVS)	sP%	p in COD	c in COD(mg/gVS)	sum of COD (mg/gVS)
1	SF	46.2	7.4	15.6	25.0	138.9	33.2	208.3	26.7	235.13
	S5	51.1	8.8	11.0	13.5	75.9	67.3	113.8	14.4	128.2
	S7	69.0	14.3	18.4	20.6	91.6	75.3	137.3	22.0	159.3
2_2	SF	17.9	1.0	2.3	19.2	160.2	11.1	240.3	20.5	260.9
	S5	150.5	7.5	14.8	18.9	200.9	74.9	301.3	20.2	321.5
	S7	180.2	11.4	22.8	26.3	226.6	79.5	339.8	28.15	368.0
3	SF	33.7	1.0	3.0	16.5	229.5	14.6	344.2	17.6	361.8
	S5	70.1	10.3	11.4	27.2	118.0	59.4	176.9	29.07	206.
	S7	116.2	12.4	16.1	30.5	173.6	66.9	260.3	32.6	293.
4	SF	18.8	1.2	4.4	15.5	149.1	12.6	223.6	16.54	240.1
	S5	31.5	13.4	17.5	20.3	60.7	51.9	90.98	21.6	112.6
	S7	260.1	11.0	25.9	29.3	299.0	87.01%	448.4	31.38845	479.8

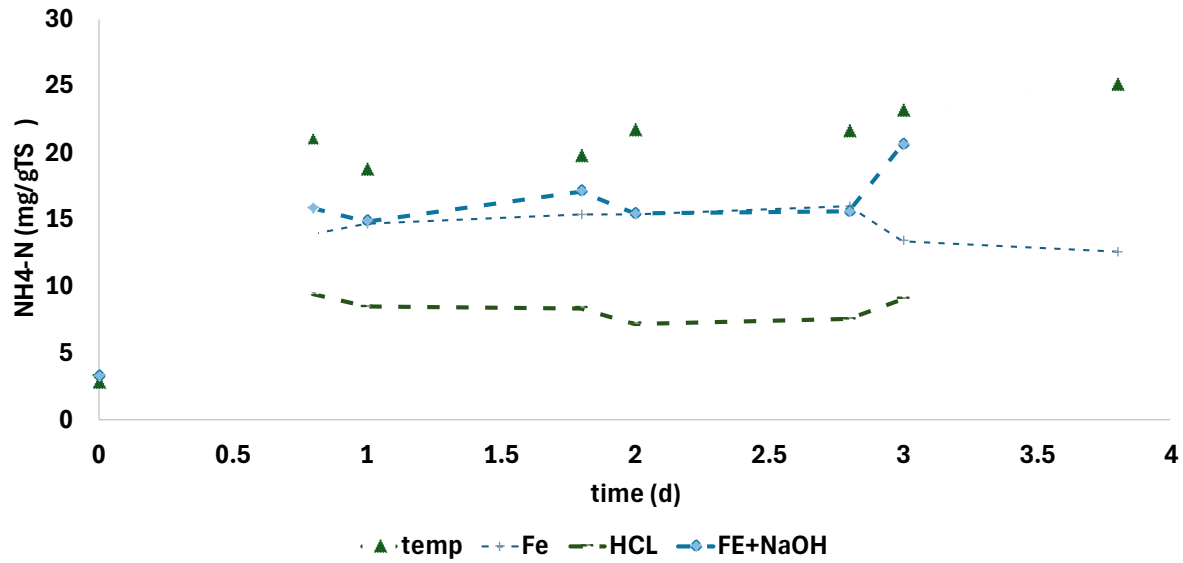
1: thermal,2: thermal with H_2O_2, 3: thermal with $FeCl_2$, 4: thermal with $FeCl_2$ and H_2O_2

SF: soluble feed, S5 : soluble at 55°C, S7 : soluble at 70°C T carbs = total carbohydrates(sum of s, LB, TB); T proteins = total proteins(sum of s, LB, TB)

Annexure 2



Annex figure 1 The sCOD release over time for the experiment with pH experiment



Annex figure 2 The ammonium release over time for the experiment with pH experiment

Annexure 3

	for 3	COD load	6000000	gm/day	VS	0.7	17892000	gCOD/day
						MW H_2	2.016	g/mol
			H_2 production		1gVS	1.42	gCOD	
Temperature			**H_2O_2 addition**	**Fe addition**	**Fe+H_2O_2**			
COD%		0.01	0.01	0.1	0.02			
Theoretical H_2 production	g/day	1699.74	1789.2	17892	3041.64			
moles	mol/day	843.125	887.5	8875	1508.75			
vol produced	L/d	18886	19880	198800	33796			
	MJ/day	204	215	2147	365			
	km/day	169.9	178.92	1789.2	304.164			
			CH_4 production					
CH_4 moles	mol/day	210.7813	221.875	2218.75	377.1875			
vol of CH_4	kg/day	3.3725	3.55	35.5	6.035			
vol of CH_4	kg/hr	0.14	0.15	1.48	0.25			
Current production	kg/hr	154	154	154	154			
%contribution	%	0.1%	1.0%	0.1%	0.2%			

Annexure 4

1mg VS = 1.42g COD

1mg COD = 350 ml of methane

experiment	Feed	70	VS	Vs in COD(g/L)	T CH_4(g)	A CH_4	ACH_4/TCH_4
Temperature	168.1	190.7	19.13	27.17	9.5	3.6	38.38%
Temp+H_2O_2	186	227.5	17.09	24.26	8.46	3.8	45.79%
Temp+$FeCl_2$	178	376.4	28.4	40.33	14.1	10.69	75.73%
Temp+$FeCl_2$+H_2O_2	211	226.	27.79	39.46	13.81	6.30	45.65%

Where A CH_4 is actual CH_4 that is produced obtained from the BMP value. TCH_4 is the CH_4 that can be produced from the VS

Actual CH_4 = BMP*VS of feed

T CH_4 = VS*1.42*350ml

The conversion percentage = (actual / theoetical) *100

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