



Enzymes targeting distinct hydrolysis blind-spots of thermal and biological pre-treatments significantly uplift biogas production[☆]

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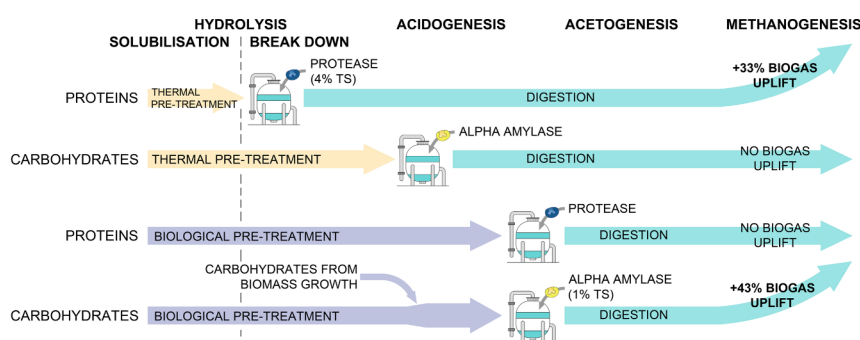
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HIGHLIGHTS

- Pre-treatments show bias towards macromolecule solubilisation.
- THP targets proteins and carbohydrates with only carbohydrates further used.
- BH targets proteins and fibres and increases the suspended carbohydrate fraction.
- Solubilisation bias creates blind spots in the hydrolysis of matrix macromolecules.

GRAPHICAL ABSTRACT



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ABSTRACT

Thermal hydrolysis process (THP) and biological hydrolysis (BH) are key pre-treatment technologies for anaerobic digestion (AD), termed advanced anaerobic digesters (AADs). They target the rate-limiting hydrolysis step in AD. This study evaluates full-scale pre-treatments for macromolecule bias and the implementation of hydrolysis enzymes to enhance biogas yield. Findings show THP significantly improves protein and carbohydrate solubilisation by 30% and 25%, respectively, but fully hydrolyses only carbohydrates. In contrast, BH targets fibres and proteins, achieving 35% and 23% solubilisation, and only partially hydrolyses carbohydrates. Bio-methane potential (BMP) tests indicate that protease enzymes raise biomethane yield by 20–30% for AAD with THP pre-treatment. In comparison, α -amylase increases it by over 30% for AAD with BH pre-treatment. This study tailors enzyme selection and dosage to specifically address the unique “hydrolysis blind spot” of each pre-treatment, providing a strategic framework to enhance AD technologies by an improved understanding of macromolecule selectivity and their transformation pathways.

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1. Introduction

Anaerobic digestion (AD) is the preferred technology for energy recovery from biomass with high moisture content, like sewage sludge, while at the same time stabilising sludge for use as organic fertiliser, mitigating greenhouse gas emissions, and removing the unpleasant odour. Conventional digesters achieve around 30 % conversion of volatile solids (VS) to biogas, with hydrolysis being the rate-limiting step encompassing the main limitations of low process efficiency and long retention times. Wastewater treatment utilities are employing pre-treatment technologies like thermal hydrolysis process (THP) and biological hydrolysis (BH) to effectively tackle these challenges associated with AD technology by engineering the rate-limiting hydrolysis step out of the main reactor. This process, known as advanced anaerobic digestion (AAD), typically increases VS destruction by up to 50 % and enables an increase in operational capacity without having to build additional AD infrastructure by allowing operation at increased organic loading rates. In thermal hydrolysis, sludge is heated to temperatures ranging from 150 °C to 170 °C and pressured at about 6–9 bar for a brief period, typically 20–30 min (Neyens et al., 2003). Following this, a rapid depressurization to atmospheric pressure occurs in a flash tank. BH, or temperature-phased anaerobic digestion, enhances hydrolysis via native enzymes at ideal thermophilic temperatures (42–70 °C) (Mehari et al., 2018). BH acts as an acid-phase fermentation pre-treatment, effectively separating the acid phase from the biogas production phase, which mainly takes place in the digester subsequent to the BH pre-treatment. Though the mechanisms of these technologies are fundamentally different, they essentially involve a reduction in particle size, solubilisation of particulate organic matter, and, in the case of BH, further conversion to volatile fatty acids (VFAs). The corresponding enhancement of methane yield is reported to be 17 and 24 % for THP (Ferrentino et al., 2019) and between 3.5–23 % for BH (Mehari et al., 2018). While this shows that AADs outperform ADs, the extent of macromolecule hydrolysis that results in the production of easily available substrates for AD microorganisms remains less clear. Thus, understanding these advancements at a mechanistic level is critical for enhancing anaerobic digestion. This knowledge gap suggests that optimisation opportunities could exist if AADs only partially hydrolyse specific macromolecules, leading to “blind spots” in their hydrolysis and conversion to VFAs. In this scenario, enhanced solubilisation, which is the first step of hydrolysis, does not necessarily translate to enhanced hydrolysis and conversion to VFAs. This phenomenon has been reported by Bougrier et al. (2006), who showed that pre-treatment with ultrasonication achieved a similar biomethane yield to thermal hydrolysis pre-treatment, even though the latter achieved roughly 3 times higher chemical oxygen demand (COD) solubilisation compared to the former.

The production of VFAs depends on the catalytic efficiency of extracellular hydrolases (Hao and Wang, 2015). Enhancing AD with hydrolysis enzymes offers benefits like mild operational conditions, ease of use, and eco-friendly characteristics owing to their biological origins (Wawrzynczyk et al., 2008). This approach allows for retrofitting existing systems without significant investment through simple dosing. Studies on enzyme addition in AD have typically focused on two methods: improving biomethane production directly (Villa et al., 2022; Donoso-Bravo and Fdz-Polanco, 2013; Recktenwald et al., 2008) or enhancing the hydrolysis phase to boost AD efficiency (Roman et al., 2006; Yang et al., 2010; Luo et al., 2012; Xin et al., 2016). The latter often targets the dissolution of suspended solids and increases in soluble macromolecules and VFAs, mainly using waste-activated sludge (WAS) due to its lower biodegradability compared to primary sludge. A study by Xin et al. (2018) combined various hydrolysis enzymes (protease, α -amylase, cellulase, lysozyme) in a 5 % (w/w) mix, reporting a 40 % increase in total biogas yield. Yu et al. (2013) utilized crude α -amylase and protease from WAS, achieving a 20 % increase in BMP with both enzymes, though α -amylase alone doubled sCOD release. This suggests that enhanced solubilisation does not guarantee increased biogas

production. Recktenwald et al. (2008) adopted a more targeted approach in enzyme selection by choosing glycosidic enzymes based on WAS *exo*-polymeric material, resulting in a 10–20 % rise in methane yield and better dewatering. The varied outcomes stem from enzyme type, test conditions, activity, and substrate nature, complicating comparisons. The economic feasibility of enzyme uses hinges on purification costs (Kendir Çakmak and Ugurlu, 2020), suggesting that lower-purity industrial-grade enzymes might be viable (Lorenzetti et al., 2020). Hydrolytic enzymes like proteases and amylases are widely utilised in industries such as detergent (Zhang et al., 2019) and biodiesel (Khan et al., 2020). Recktenwald et al. (2008) showed the economic viability of glycosidic enzymes in full-scale AD with enhanced biogas and dewatering. Some studies found enzyme mixtures from food waste (Kim et al., 2023) or crude enzymes from bacterial cultures (Pinheiro et al., 2022) cost-effective. However, these do not account for specific enzyme dosing based on digester needs, which vary with substrate and conditions. No research has yet explored the synergistic effects of hydrolytic enzymes tailored to pre-treated sludge.

Hence, two hypotheses are put forth in this study. 1) AADs have certain blind spots in the solubilisation and hydrolysis of macromolecules, depending on their working mechanism. 2) The addition of hydrolysis enzymes of industrial nature can be tailored to complement existing pre-treatment technologies by targeting these hydrolysis blind spots.

This study is novel in its exploration of the bias in macromolecule solubilisation and further hydrolysis in AAD to boost biomethane yield by adding hydrolysis enzymes.

2. Materials and methods

2.1. Sample collection and characteristics

The pre-treatments chosen for this study are from two different full-scale sites in the UK treating municipal sewage sludge generated from primary and secondary settling tanks. The first site employs THP pre-treatment from CAMBI at 160 °C and 6 bar for 30 min, followed by a flash tank. The second site employs biological pre-treatment, a three-stage process involving heat recovery, pasteurisation, and biological hydrolysis. During the heating stage, the temperature of the raw sludge is increased to 40–45 °C using the hot water recovered from the Combined Heat and Power (CHP) engines. The sludge is then alternately transferred to two pasteurisation tanks with a retention time of 5 h, where it is pasteurised at 55 °C for 5 h. Finally, the sludge is transferred to a hydrolysis tank, cooled to a temperature of 38–42 °C, and held for typically 1.5 days to achieve hydrolysis before being fed to the digesters. The THP-AD and BH-AD sites serve a population equivalent of 2.57 and 1.49 million, respectively. Both sites receive 60 % of primary sludge, 20 % of WAS and 20 % of sludge imports, although the fraction of sludge imports can vary between 0 and 20 %. The sludge imports are from decentralised wastewater treatment sites, generally with primary and fixed biofilm processes as core treatment units. Neither of the sites receives industrial sludges. For analysis of macromolecules, the sludge was collected just once as the study aimed to understand how macromolecules are impacted based on pre-treatment type rather than capturing temporal variance. For this objective, the sludge samples were collected before pre-treatment (raw sludge), after pre-treatment (pre-treated sludge) and after digestion (digestate). For the BMP tests, the pre-treated sludge (substrate) and digestate (inoculum) were characterised for total solids (TS), volatile solids (VS) and pH (Table 1). All sludge samples were stored at 4 °C and processed either for macromolecule analysis or used in BMP tests within a week of their collection.

Table 1

Characteristics of sludge samples collected from the two full-scale sites used for BMP tests.

	TS (mg/L)	VS (mg/L)	pH
THP-AD site			
Substrate	87 ± 8	66 ± 7	5.34 ± 0.06
Inoculum	55 ± 8	35 ± 3	8.12 ± 0.06
BH-AD site			
Substrate	77 ± 5	60 ± 7	5.66 ± 0.11
Inoculum	50 ± 6	33 ± 5	8.67 ± 0.50

*TS = total solids; VS = volatile solids.

Note: Samples analysed in triplicates.

2.2. Sludge characterisation

2.2.1. Separation and quantification of suspended sludge fraction for macromolecule characterisation

To conduct a preliminary investigation of the effects of thermal and biological pre-treatments on the solubilisation of macromolecules, the suspended fraction was separated by bench-scale centrifugation of the sludge samples. The sludge was first centrifuged in 50 mL tubes at 4,696 g and 19 °C for 30 mins. The supernatant was discarded, and the pellet was resuspended with DI water to its original volume and centrifuged again for another 30 min to remove any residual dissolved fraction. The supernatant from centrifugation was again discarded, and the suspended fraction of sludge (i.e. pellet) was flushed out with DI from the tubes and into shallow aluminium pans, ensuring the thickness of the sample did not exceed 2 cm. The samples were then oven-dried at 55 °C for 2 days until completely dried. A low temperature was adopted to enable drying without causing the macromolecules to disintegrate. This dried sample, referred to as the suspended fraction of the original fresh sludge, was used for further macromolecules analysis (suspended macromolecules) for proteins, lipids, fibres and carbohydrates.

2.3. Biomethane potential tests

BMP tests were conducted in 1L reactors, with a 2:1 inoculum : substrate ratio on a VS basis at 40 °C using CJC (UK) and Bioprocess Control (BPC-AMPTS-II) (Sweden) automated systems with 30 % headspace. Five enzymes (protease, alcalase, cellulase, lipase, α-amylase) were chosen (Table 2) to target different macromolecules in the sludge and dosed at 0.5 %, 1 %, 2 % and 4 % total solids (TS) of feed directly into the batch reactors with the substrate and inoculum. A control group with just the substrate and inoculum was operated without any enzyme addition, and an equivalent volume of inoculum and deionised water was operated instead of the substrate to serve as the blank group. This was done to get the biogas produced from inoculum alone while keeping all other reactor conditions like headspace and working volume similar to control and test groups. CH₄ concentration in

the biogas was determined daily via gas chromatography (Valco Instruments Company Inc., USA). Biogas and CH₄ yields (mL/g VS added of feed) were calculated from their accumulated production in mL after subtracting the accumulated biogas production from the blank and normalising to the mass of VS from the sewage sludge fed to the reactor. All units for gas production are normalised to standard temperature and pressure (0 °C and 1 atm) with no moisture. Digestion ended when daily biogas production increased by less than 1 % for three consecutive days (Holliger et al., 2016). All experiments were conducted in triplicates, except for the control and blank, which were conducted in duplicates, and average values were reported with standard deviations.

2.4. Kinetic models

First-order kinetics were used to estimate biogas production kinetics, with equation (1):

$$C = C_{\max} \times (1 - \exp(-k \times t)) \quad (1)$$

where C is the cumulative CH₄ production (mL CH₄/g VS), $C_{\max}$ is the maximum CH₄ production potential (mL CH₄/g VS), k is the first-order hydrolysis rate constant (d⁻¹), and t is the duration of the AD process (d).

All parameters were calculated using non-linear regression using SPSS software. R-square (Equation (2)) was used as an indicative value of the quality of the model fit.

$$R^2 = \left[\frac{m(\sum_j^m X_j Y_j) - (\sum_j^m X_j)(\sum_j^m Y_j)}{\sqrt{[m\sum_{j=1}^m X_j^2 - (\sum_{j=1}^m X_j)^2][\sum_{j=1}^m Y_j^2 - (\sum_{j=1}^m Y_j)^2]}} \right]^2 \quad (2)$$

Where Y_j is the measured value, and X_j is the predicted value by the model. An R^2 value of 1 shows a perfect fit for the model, while a value of 0 shows no correlation between the measured and predicted models.

2.5. Analytical methods

Physical characteristics (TS and VS) of all the collected sludge samples were analysed following standard protocols (APHA, 1999). To determine the concentration of the suspended solids fraction of all samples by the centrifugation method, the final pellet obtained was dried in the centrifuge tubes at 55 °C for 2 days. The tubes were weighed two consecutive times, spaced by 2 h, and were considered fully dry when the values attained at both times remained constant. The concentration of the suspended fraction was then calculated using equation (3).

$$F_{ss} = \frac{(W_{tss} - W_t)}{(W_{ts} - W_t)} \times 1000 \left(\frac{g}{L} \right) \quad (3)$$

Table 2

Description of industrial grade enzymes used in the study.

Enzyme name	Commercial name	Activity	Description	Optimum temperature (°C)	Optimum pH range	Company
Cellulase from <i>Aspergillus N.</i>	Carezyme	1000 U/g	Catalyses the hydrolysis of cellulose by randomly splitting the sugar residues within the molecule.	25–35	5–8	Sigma
Lipase from <i>Rhizopus oryzae</i>	Lipase	10,000 FIP/g	Catalyses the hydrolysis of fats and oil.	up to 50	3–9	Biocon
Subtilisin	Alcalase	2.5 AU/g	Catalyses the hydrolysis of proteins with a broad specificity towards native and denatured proteins and is active under alkaline conditions.	30–65	7–10	Novozymes
Protease from <i>Bacillus Licheniformis</i>	Protease	1000 UB/g	Broad-spectrum protease mixture used to liquefy mucins and digest proteins to free amino acids.	up to 60	5–10.5	Biocon
α-amylase from <i>Bacillus Licheniformis</i>	α-amylase	4000 U/g	Catalyses the hydrolysis of polysaccharides	up to 100	5–10	Biocon

Note: U = unit; FIP = Fédération Internationale Pharmaceutique; AU = Anson unit; UB = Unit branching activity.

Where F_{SS} is the fraction of the suspended solids, W_{ISS} is the weight of the centrifuge tube with the dried suspended fraction after drying at 55 °C for 2 days, W_t is the weight of the centrifuge tube alone and W_{is} is the weight of the centrifuge tube with the sludge sample before the centrifuge step. The volatile fraction of suspended solids was then determined following the same standard protocol for VS (APHA, 1999).

For analysis of proteins, a portion of the dried and blended sludge was further dried at 105 °C for 2 h to remove any trace amounts of moisture and its total nitrogen concentration was quantified using a Shimadzu Total nitrogen analyser. To account for any residual ammonia and nitrite after the drying step at 105 °C, 1 g of dried sludge was resuspended in 10 mL of DI water and shaken in a linear shaker for 10 min. The resuspended solution was then centrifuged at 4,696 g for 10 min, and the supernatant was tested for residual ammonia and nitrite. The amount of protein was then calculated by subtracting the residual total ammonia nitrogen (TAN) and nitrite values from the total nitrogen value obtained from the elemental analyser and multiplying the result with the conversion factor of 6.25 based on the assumption that all proteins had a nitrogen content of 16 % (Moore et al., 2010). The lipid content was quantified from the dried suspended fraction using a DIONEX Accelerated Solvent Extractor (ASE) 200 with a solvent mixture of 60 % hexane, 20 % acetone and 20 % methanol (Dufreche et al., 2007). The crude fibre content was quantified using the method described by Van Soest et al. (1991). The carbohydrate content was then quantified using the mass balance calculations after determining the fibres, lipids and proteins content of the volatile fraction of suspended solids.

The pH measurement was carried out with a Mettler Toledo (UK) MA235 pH/ion analyser and a portable Hanna HI 991003 pH meter. Soluble COD and TAN were analysed using cell test kits from Millipore (VWR, UK) and a spectrometer Spectroquant NOVA 60 from the same company. For soluble COD and TAN analysis, the solids-free fraction of the samples was used, which was generated by subjecting the samples to centrifugation in a Megafuge 16R centrifuge (Thermo Scientific, Massachusetts, USA) for 20 min at 4,696 g and 19 °C and filtering the supernatant with a 0.45 µm syringe filter (Whatman, Kent, UK). The filtrates were then stored at 4 °C and analysed within 2 days of cold storage.

2.6. Statistical analysis

Student's T test using excel was used to assess whether the difference between the control and test groups was statistically significant or not, with the result considered significant if the p-value was less than 0.05.

3. Results and discussion

3.1. Impact of thermal and biological pre-treatments on sludge macromolecule composition

Suspended macromolecules were profiled to shed light on the extent of solubilisation induced by the pre-treatments on proteins, carbohydrates, lipids, and fibres. The analysis also aimed to determine whether any inherent bias exists in the solubilisation of these macromolecules. The results presented are a snapshot of macromolecule profiles across the site with the intention of capturing the impact of pre-treatments on macromolecule composition rather than profiling these changes with time. Accounting for the difference in total solids distribution in both the sites, the results were normalised to gVS for a more impartial comparison of macromolecule fate. The raw sludge collected before pre-treatment from both sites had a similar composition of macromolecules in the suspended fraction, with protein being the dominant fraction. The protein concentrations were 298 ± 1 and 314 ± 1 mg/gVS at the THP-AD and BH-AD sites, respectively (Fig. 1). However, the concentration of TAN in the raw sludge before pre-treatment was much higher in the BH-AD site (1107 ± 23 mg/L) as compared to the THP-AD site (622 ± 33 mg/L). This is because most of the imported sludge at the BH-AD site comes from small-scale wastewater treatment plants where secondary biological treatment only targets the removal of biological oxygen demand (BOD), resulting in high ammonia levels. THP pre-treatment significantly reduced the suspended protein fraction from 298 ± 1 mg/gVS to 208 ± 3 mg/gVS, representing a 30 ± 4 % solubilisation (T-test, p-value < 0.05). It also significantly reduced the suspended carbohydrate fraction from 194 ± 13 mg/gVS to 145 ± 11 mg/gVS, representing a 25 ± 9 % solubilisation (T-test, p-value < 0.05) while having a negligible effect on fibres (Fig. 1). In contrast, BH pre-treatment significantly reduced the suspended fraction of fibres from 225 ± 17 mg/gVS to 146 ± 6 mg/gVS, representing a 35 ± 8 % solubilisation (T-test, p-value < 0.05). It also reduced the suspended fraction of proteins from 314 ± 5 mg/gVS to 242 ± 2 mg/gVS, representing a 23 ± 2 % solubilisation (T-test, p-value < 0.05). These results highlight the difference in macromolecule selectivity between biological pre-treatments and thermal pre-treatments. Both pre-treatments doubled the sCOD fraction. However, BH pre-treatment nearly doubled TAN concentrations from 1107 ± 23 to 2128 ± 17 mg/L. This contrasts with the negligible increase in ammonia levels post-THP pretreatment after accounting for feed dilution after pre-treatment from 184 ± 7 gTS/L to 79 ± 5 gTS/L with tertiary treated effluent. Therefore, after accounting for the feed dilution post-THP, the TAN increases from 622 ± 33 to 822 ± 107 mg/L, corresponding to an increase of 32 ± 18 % of feed TAN. This

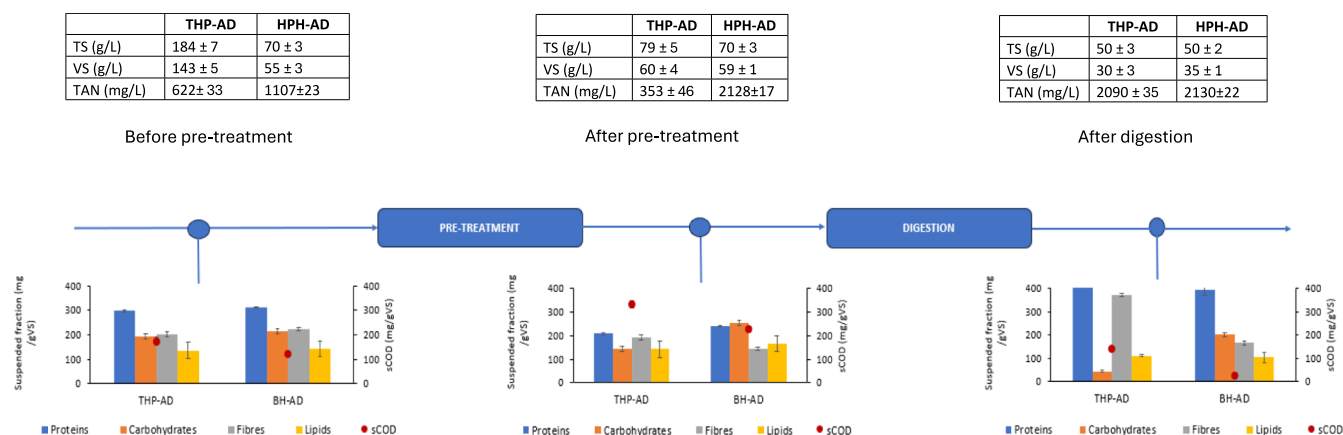


Fig. 1. Distribution of suspended macromolecules fractions and sCOD across the sludge treatment chain in the studied full-scale THP- and BH-AD sites indicated along with solids characterisation and TAN levels.

suggests that BH can achieve enhanced hydrolysis of proteins after initial solubilisation, while THP enhances protein solubilisation but not full hydrolysis, which takes place in the AD itself. The results correlate with findings from [Hung-Wei Lu et al. \(2014\)](#), who investigated the effect of THP temperature and time on the solubility of organic solids. They reported that the solubility of carbohydrates occurs below 150 °C, above which the solubility of proteins becomes more evident. Furthermore, in a study by [Wilson and Novak \(2009\)](#) that investigated THP effects on macromolecule hydrolysis in primary and WAS, commonly employed temperatures between 150 and 170 °C were observed to enhance protein solubilisation with only little subsequent hydrolysis to peptides and amino acids.

Conversely, [Liu and Chang \(2023\)](#) reported that BH influenced protein hydrolysis the most, which made a major contribution to VFA production post-pre-treatment. However, the present study shows an opposing effect on carbohydrate solubilisation, as the suspended carbohydrate fraction increased from 216 ± 7 mg/gVS in the raw sludge to 253 ± 11 mg/gVS after pre-treatment, representing an increase of 17 ± 6 % (T-test, p -value < 0.05). Given that BH is a biological pre-treatment where conditions are optimised for bacteria to break down complex macromolecules, bacterial growth is often accompanied by the production of extracellular polymeric substances (EPS) ([Garnier et al., 2005](#)). These are the construction materials of microbial aggregates in activated sludge flocs or biofilms and include different classes of macromolecules such as polysaccharides, proteins, nucleic acids, (phospho) lipids and other polymeric compounds which fill and form the space between microbial cells in the aggregate ([Garnier et al., 2005](#)). EPS production is highly dependent on the culture conditions of pH and temperature ([Zisu and Shah, 2003](#)). Studies have found EPS production to be highest at a pH between 5 and 7, which is the pH range for the acidogenic phase within AD ([Sørensen et al., 2022](#)). [Liu and Chang \(2023\)](#) found no significant change in volatile suspended solids (VSS) after 3 days of BH and concluded that the ultimate VSS reduction that can be achieved by BH treatment will eventually depend on the balance between biomass decay and growth of anaerobic bacteria. [Liu and Fang \(2002\)](#) observed that carbohydrate was a major EPS component in acidogenic sludge (62 % w/w of EPS), while protein was a major EPS component in methanogenic sludge (41 % w/w of EPS). BH constitutes most of the acidogenic phase of AD, with the resulting pre-treated sludge being acidogenic. It is hence postulated that the increase in the carbohydrate fraction observed in the present study could be the result of an increase in the EPS fraction after pre-treatment. Hence, it is evident that the feed sludges going into the digesters have a different macromolecular makeup defined by the type of pre-treatment employed. For the BH site, the suspended fraction of the pre-treated sludge mostly consisted of proteins and carbohydrate fractions relative to other macromolecules. For the THP site, this fraction mostly consisted of proteins and fibres (in relative proportion to other macromolecules) ([Fig. 1](#)).

The digestates from the two sites were also distinct in their macromolecule profile. The suspended fraction of digestate from the THP-AD site was almost equally dominated by proteins and fibres at 412 ± 7 and 372 ± 6 mg/gVS, respectively, while that from the BH site was dominated mostly by proteins at 394 ± 26 mg/g VS, followed by carbohydrates at 201 ± 9 mg/gVS. The digestate from the THP-AD site had a significantly lower suspended carbohydrate fraction (45 ± 5 mg/gVS) compared to the BH-AD site (201 ± 9 mg/gVS) indicating that THP pre-treatment had a greater impact on the solubilisation of carbohydrate fraction. This finding agrees with previous studies showing that THP significantly enhances the solubility of carbohydrates by breaking down complex polysaccharides into smaller, more easily digestible molecules ([Carranza Muñoz et al., 2024](#); [Shana et al., 2013](#)). Conversely, the suspended fraction of fibres after digestion with BH-pre-treatment (166 ± 11 mg/gVS) was almost half of its thermal counterpart (372 ± 6 mg/gVS), indicating that BH had a greater impact on the solubilization of fibres in the digester over THP pre-treatment. It is hypothesised that BH pre-treatment enriches hydrolytic bacteria in the digester that breaks

down fibres such as cellulose, the predominant fraction of fibres in sewage sludge. Hence the suspended fraction of digestate with THP pre-treatment predominantly consisted of proteins and fibres, almost in equal proportion, with the carbohydrate fraction largely diminished. However, the suspended fraction of digestate with BH pre-treatment predominantly composed of proteins, followed by carbohydrates and fibres in nearly equal proportions ([Fig. 1](#)).

With regards to the soluble organics in the digestate, digestate from THP-AD had a much higher residual sCOD (142 ± 3 mg/gVS), which is more than 5.7 times greater than that from BH-AD digestate (25 mg/gVS). Given the similar sludge retention time in both the sites, this suggests that THP-pre-treatment enhanced the solubilisation of macromolecules, but compared to BH pre-treatment, a greater part of this solubilised fraction was not converted to biogas in the digester. Moreover, the high residual sCOD in the digester is also attributed to the presence of refractory melanoidins formed through the Maillard reaction in the THP-AD ([Cao et al., 2023](#)). This is in agreement with findings from [Ferrentino et al. \(2019\)](#), who reported that around 50 % of the soluble COD was still detected in the digestate from THP pre-treated sludge after 23 days of digestion. They concluded that not all the solubilised organics were used for biogas production. Similarly, [Davidsson and Jansen \(2006\)](#) showed that thermal pre-treatment of WAS at 70 °C achieved 19 % COD solubilisation compared to just 1 % achieved by ultrasonication. However, the BMP of the ultrasonicated WAS was still approximately 4 % higher than that of the thermally pre-treated WAS, suggesting an imbalance between the enhanced solubilization and biogas production. The conversion efficiency of solubilised organics to biogas depends on many factors like the type of organic substances ([Bougrier et al., 2006](#)), microbial communities ([Pasalari et al., 2021](#)), operational conditions ([Wahid and Horn, 2021](#)) and presence of inhibitory substances ([Chen et al., 2008](#)), all of which are influenced by pre-treatment ([Liu and Chang, 2023](#)). Despite these results, another aspect of thermal hydrolysis pre-treatment must be considered: the volatilisation of organics due to the high temperature followed by flashing. [Ngo et al. \(2023\)](#) observed a significant decrease in TS by 24.6 % and an increase in VS as %TS from 78 % to 87 % at bench-scale after thermal hydrolysis at 145 °C and 10.2–14.1 bar pressure followed by flashing. Their study implies that some of the solubilised organics and inorganics are volatilised to the reactor headspace. In a Cambi system, the outlet gas from THP is recondensed and sent to the digester, negating any VS loss due to thermal pre-treatment in the overall treatment chain. Consequently, for THP-AD, the concentration and composition of soluble organics are dictated not just by the pre-treatment mechanism but also by system configuration.

3.2. Synergies of hydrolysis enzymes with thermal hydrolysis pre-treatment

The addition of enzymes targeting proteins resulted in a significant uplift in the biomethane yield of THP-pre-treated sludge even at the lower dose ([Table 3](#)). This links to the previous findings on enhanced protein solubilisation after THP pre-treatment but lower conversion to VFAs. The BMP results with protease addition revealed that a significant portion of the soluble fraction resulting from THP remains unutilised in the digester, offering opportunities for optimisation by enzyme dosing. The average methane concentration in the biogas ranged between 68 and 71 % and did not vary significantly between control and test reactors for all the enzymes tested. Protease at 0.5 % (5 UB/gTS), 1 % (10 UB/gTS), 2 % (20 UB/gTS), and 4 % (40 UB/gTS) TS of feed (w/w) resulted in an increase in BMP of 16 ± 3 %, 20 ± 3 %, 29 ± 2 % and 33 ± 5 % respectively, suggesting a positive response with increasing dose ([Table 3](#)). Alcalase enzyme, also targeting proteins, showed significant increases in BMP by 22 ± 4 %, 20 ± 2 %, 21 ± 3 % and 22 ± 2 % at 0.5 % (12.5 AU/gTS), 1 % (25 AU/gTS), 2 % (50 AU/gTS) and 4 % (100 AU/gTS) TS (w/w) respectively and was consistent in enhancing the BMP irrespective of the dose applied. Augmenting THP-AD with α -amylase

Table 3

BMP (NmLCH₄/g VS fed) of THP-pretreated sludge with enzymes addition to the batch ADs compared to control sludge AD (without enzyme addition).

Enzyme	Enzyme dose as % of TS (w/w)				
	0 (Control)	0.5 %	1 %	2 %	4 %
Protease	456 ± 8	529 ± 10 (↑16 ± 3 %)	550 ± 12 (↑20 ± 3 %)	590 ± 3 (↑29 ± 2 %)	607 ± 19 (↑33 ± 5 %)
Alcalase	408 ± 5	495 ± 15 (↑22 ± 4 %)	489 ± 6 (↑20 ± 2 %)	492 ± 12 (↑21 ± 3 %)	497 ± 5 (↑22 ± 2 %)
α-amylase	426 ± 30	383 ± 18	374 ± 16	429 ± 13	456 ± 19
Cellulase	407 ± 5	388 ± 4	384 ± 8	384 ± 8	380 ± 2
Lipase	426 ± 30	371 ± 4	394 ± 13	398 ± 7	383 ± 3

Note:

- Figures in brackets indicate a significant impact on BMP with p-value < 0.05, while those without bracketed values were not statistically different from control reactors.
- Methane volume normalized to 0 °C, 1 atm, and no moisture.
- All samples, except the control, are in triplicate. The control is in duplicate.

did not enhance the methane yield, which indicates that THP already achieves enhanced solubilisation and utilisation of carbohydrates in the digester, as evidenced by the low fraction of VS attributed to carbohydrates in the full-scale macro-composition analysis (Fig. 1).

The analysis of macromolecule composition in sludge from full-scale sites revealed that THP improved the solubilisation of proteins and carbohydrates in the pre-treated feed. BMP test results indicated that protease and alcalase, targeting proteins, increased biomethane yield. In contrast, α-amylase, which targets carbohydrates, had no effect. Since all three enzymes operated under optimal conditions (Table 2), this suggests that THP-AD effectively hydrolysed carbohydrates, while the hydrolysis of proteins was only partial and mostly involved solubilisation. Thus, supplementing THP-AD with protease enzymes could enhance the hydrolysis of the protein fraction that the digester only partially hydrolyses, subsequently boosting methane yield.

Kinetic studies indicate a good fit with first-order kinetics, having an R² value > 0.98 (Table 4). The k value is a first-order rate constant, with a higher k value indicating a higher substrate hydrolysis rate. The hydrolysis rate of all reactors dosed with protease, α-amylase and lipase were like those of their respective control reactors, despite the significantly higher BMP values with protease enzyme. This shows that the increase in BMP with protease resulted from expanding the existing pool of hydrolysed substrates with no influence on hydrolysis reaction kinetics. In other words, the addition of protease enzyme enhances the hydrolysis potential of the substrate rather than its reaction rate. However, the hydrolysis rate (k values) with alcalase and cellulase at different doses were lower than for the control ADs by 18–23 % and 6–12 %, respectively, for all the doses studied (T-test, p-value < 0.05). For alcalase, this suggests that while it enhances hydrolysis potential, it may do so at the expense of the hydrolysis rate. The reduced kinetics could be due to an accumulation of hydrolysis products, which may either inhibit the AD process or cause a lag phase as microbes adapt to the increased substrate availability. The relationship between enzymatic pre-treatment and reaction kinetics remains insufficiently explored, highlighting the need for further investigation.

3.3. Synergies of hydrolysis enzymes with biological hydrolysis pre-treatment

Contrary to the results of THP-AD, batch tests with enzyme dosing on BH pre-treated sludge showed a significant uplift in BMP with carbohydrate-targeting α-amylase (Table 5). α-amylase at 0.5 % (20 U/

Table 4

First-order kinetic constants for BMP tests with the addition of hydrolysis enzymes for THP pre-treated sludge.

Enzyme	Enzyme dose as % TS (w/w)				
	0 % (Control)	0.5 %	1 %	2 %	4 %
C_{max} (NmL CH₄/g VS added)	461 ± 11	529 ± 10	549 ± 13	592 ± 5	612 ± 19
k (d⁻¹)	0.327 ± 0.008	0.333 ± 0.008	0.323 ± 0.004	0.3 ± 0.01	0.28 ± 0.014
R²	0.981 ± 0.002	0.992 ± 0.001	0.996 ± 0.002	0.999 ± 0	0.999 ± 0.001
Alcalase	0 % (Control)	0.5 %	1 %	2 %	4 %
C_{max} (NmL CH₄/g VS added)	396 ± 5	486 ± 15	479 ± 8	482 ± 2	490 ± 16
k (d⁻¹)	0.476 ± 0	0.387 ± 0.007	0.392 ± 0.008	0.379 ± 0.014	0.367 ± 0.012
R²	0.991 ± 0.001	0.995 ± 0.001	0.996 ± 0.001	0.998 ± 0.001	0.998 ± 0.003
Cellulase	0 % (Control)	0.5 %	1 %	2 %	4 %
C_{max} (NmL CH₄/g VS added)	392 ± 4	378 ± 4	373 ± 8	375 ± 9	376 ± 8
k (d⁻¹)	0.547 ± 0.003	0.481 ± 0.004	0.493 ± 0.007	0.502 ± 0.019	0.513 ± 0.02
R²	0.99 ± 0.001	0.993 ± 0.005	0.99 ± 0.001	0.992 ± 0.001	0.991 ± 0.001
Almpha amylase	0 % (Control)	0.5 %	1 %	2 %	4 %
C_{max} (NmL CH₄/g VS added)	421 ± 30	378 ± 14	371 ± 16	420 ± 14	454 ± 18
k (d⁻¹)	0.376 ± 0.015	0.401 ± 0.047	0.405 ± 0.053	0.406 ± 0.01	0.359 ± 0.007
R²	0.991 ± 0.003	0.991 ± 0.003	0.993 ± 0.002	0.992 ± 0.001	0.994 ± 0.002
Lipase	0 % (Control)	0.5 %	1 %	2 %	4 %
C_{max} (NmL CH₄/g VS added)	421 ± 30	367 ± 5	392 ± 13	390 ± 8	378 ± 3
k (d⁻¹)	0.376 ± 0.015	0.413 ± 0.009	0.394 ± 0.02	0.414 ± 0.013	0.414 ± 0.02
R²	0.991 ± 0.003	0.989 ± 0.001	0.99 ± 0.002	0.991 ± 0.002	0.992 ± 0.001

Note:

- All samples, except the control, are in triplicate. The control is in duplicate.
- Methane volume normalized to 0 °C, 1 atm, and no moisture.

Table 5

BMP (NmLCH₄/g VS fed) of BH-pretreated sludge with enzymes added to the batch ADs compared to control sludge AD (without enzyme addition).

Enzyme	Enzyme dose (%TS) (w/w)				
	0 (Control)	0.5 %	1 %	2 %	4 %
Protease	364 ± 12	376 ± 4	377 ± 4	381 ± 9	389 ± 7
Alcalase	393 ± 6	391 ± 11	390 ± 13	412 ± 9	399 ± 13
Cellulase	360 ± 20	386 ± 6	377 ± 16	409 ± 4	393 ± 15
α-amylase	240 ± 23	326 ± 27 (↑36 ± 15 %)	343 ± 17 (↑43 ± 13 %)	342 ± 35 (↑42 ± 18 %)	321 ± 16 (↑33 ± 12 %)
Lipase	249 ± 11	259 ± 9	244 ± 18	255 ± 15	235 ± 7

Note:

- Figures in brackets indicate a significant impact on BMP with p-value < 0.05, while those without bracketed values were not statistically different from control reactors.
- Methane volume normalized to 0 °C, 1 atm, and no moisture.
- All samples, except the control, are in triplicate. The control is in duplicate.

gTS), 1 % (40 U/gTS), 2 % (80 U/gTS) and 4 % (160 U/gTS) TS (w/w) dose resulted in an increase in BMP of 36 ± 15 %, 43 ± 13 %, 42 ± 18 % and 33 ± 12 % respectively with a p-value < 0.5 (T-test) (Table 5). Previous results on sludge macromolecule characterisation on the full-scale sites showed a significant increase in the suspended fraction of carbohydrates after BH pre-treatment by 17 ± 6 %. In conjunction with these results, it is possible that the observed enhanced biomethane yield was a result of the α -amylase enzyme targeting the suspended carbohydrate fraction in the digester receiving BH-pre-treated sludge. Previous studies have also reported a cascading effect on the degradation of macromolecules through quorum sensing mechanisms (He and Zhu, 2024), implying that the addition of α -amylase enzyme could result in the degradation of additional macromolecules other than the macromolecule specifically targeted by the enzyme dosed, in this case, carbohydrates. This merits further investigation to elucidate the mechanism behind the significant biogas uplifts recorded.

The enzymes targeting proteins (protease and alcalase) did not affect BMP, which differs from their enhanced performance with THP pre-treated sludge. This indicates that BH already achieves enhanced protein hydrolysis and acidification, evidenced by 1.8 times the increase in ammonia concentration from 1107 ± 23 mg/L in the sludge before pre-treatment to 2128 ± 17 mg/L in the pre-treated sludge. These results correlate with findings from Liu and Chang (2023) who reported that the EPS proteins were the significant fraction of proteins hydrolysed in BH pre-treatment. These proteins were readily biodegradable and contributed the most towards VFA production. Furthermore, while investigating the relationships between the different digestate parameters in BH-AD, Wang et al. (2022) found a strong correlation between residual sCOD, humic acids, and polysaccharides in the digestate, while proteins correlated weakly to residual sCOD. Their study further implies that soluble proteins are more efficiently used in BH-AD than other macromolecules. BMP with cellulase enzyme also did not have any impact on enhancing BMP. Given the enhanced solubilisation of fibres observed during the characterisation of suspended fraction, it can be concluded that BH pre-treatment enabled both solubilisation and hydrolysis of fibres in the digester, leaving little room for positive impact when dosing fibre-targeting enzymes. It is also postulated that enzymes do not easily degrade the suspended fraction of fibres as they may also consist of hard to degrade forms such as hemicellulose and lignin.

The methane concentration in the biogas ranged between 62 and 69 % for all tests and did not significantly differ from control ADs (65–68 %). An exception to this was for lipase, which showed a significantly higher (T-test, p-value < 0.05) biogas production at 28 % and 22 % when dosing 1 % (250 FIP/gTS) and 2 % (500 FIP/gTS) TS fed (w/w) of the enzyme, respectively, but sustained a lower methane content of 53–58 % compared to control at 62–63 %. Hence, there was no significant increase in the methane yield of BH pre-treated sludge with lipase enzyme. It is hypothesised that the downstream AD processes could be inhibited due to the accumulation of lipid hydrolysis products, although this warrants further investigation. An increase in temperature, such as those experienced in a BH pre-treatment, can lead to bacteria producing lipids with longer chain lengths and higher degrees of unsaturation to adjust membrane fluidity (Sollich et al., 2017). These unsaturated lipids, containing double bonds, are generally easier to degrade by bacteria compared to saturated lipids due to reactive sites that can be targeted by enzymes for breakdown (Ali and Ibrahim, 2022; Torres et al., 2021). From these studies, it can be postulated that the increased degree of lipid unsaturation led to greater enzyme accessibility, resulting in enhanced hydrolysis of the BH pre-treated sludge. At the same time, the hydrolysis products of lipids are long-chain fatty acids (LCFAs) that have been reported to inhibit acetogens and methanogens in AD operated in a semi-continuous mode (Rodríguez-Méndez et al., 2017). This could explain the reduced methane content in the biogas of reactors dosed with lipase enzymes.

The biomethane generated from BH-AD showed a good fit with first-order kinetics, having R^2 values greater than 0.98 (Table 6). Lipase and

Table 6

First-order kinetic constants for BMP tests with the addition of hydrolysis enzymes for BH pre-treated sludge.

Enzyme	Enzyme dose as %TS				
Protease	0 %	0.5 %	1 %	2 %	4 %
	(Control)				
C_{max} (NmL CH₄/g VS added)	354 ± 9	367 ± 4	369 ± 5	373 ± 9	385 ± 7
k (d⁻¹)	0.456 ± 0.001	0.436 ± 0.006	0.409 ± 0.009	0.409 ± 0.014	0.336 ± 0.01
R²	0.995 ± 0.003	0.995 ± 0	0.996 ± 0.001	0.998 ± 0.001	0.999 ± 0
Alcalase	0 %	0.5 %	1 %	2 %	4 %
	(Control)				
C_{max} (NmL CH₄/g VS added)	388 ± 6	387 ± 10	386 ± 13	406 ± 9	394 ± 13
k (d⁻¹)	0.507 ± 0.005	0.527 ± 0.01	0.523 ± 0.011	0.493 ± 0.014	0.489 ± 0.008
R²	0.998 ± 0	0.998 ± 0.001	0.998 ± 0.001	0.998 ± 0.001	0.998 ± 0.001
Cellulase	0 %	0.5 %	1 %	2 %	4 %
	(Control)				
C_{max} (NmL CH₄/g VS added)	362 ± 21	384 ± 7	374 ± 15	404 ± 3	395 ± 16
k (d⁻¹)	0.37 ± 0.005	0.445 ± 0.004	0.425 ± 0.024	0.416 ± 0.017	0.425 ± 0.013
R²	0.986 ± 0.001	0.988 ± 0.001	0.99 ± 0.001	0.992 ± 0.002	0.985 ± 0.002
Alpha amylase	0 %	0.5 %	1 %	2 %	4 %
	(Control)				
C_{max} (NmL CH₄/g VS added)	238 ± 23	326 ± 28	342 ± 21	338 ± 35	322 ± 16
k (d⁻¹)	0.58 ± 0.008	0.53 ± 0.02	0.527 ± 0.021	0.555 ± 0.036	0.542 ± 0.023
R²	0.989 ± 0.003	0.995 ± 0	0.994 ± 0.002	0.994 ± 0.004	0.991 ± 0.002
Lipase	0 %	0.5 %	1 %	2 %	4 %
	(Control)				
C_{max} (NmL CH₄/g VS added)	248 ± 11	258 ± 10	243 ± 18	254 ± 17	236 ± 7
k (d⁻¹)	0.571 ± 0.001	0.659 ± 0.015	0.775 ± 0.065	0.803 ± 0.084	0.718 ± 0.012
R²	0.987 ± 0.001	0.998 ± 0.001	0.997 ± 0.002	0.997 ± 0.001	0.996 ± 0.002

Note:

1. All samples, except the control, are in triplicate. The control is in duplicate.
2. Methane volume normalized to 0 °C, 1 atm, and no moisture.

cellulase enzymes significantly increased the hydrolysis rate constant (k) (p value < 0.05) by 15 %, 26 %, 41 % and 26 %; and by 20 %, 15 %, 12 % and 15 %, respectively at 0.5 %, 1 %, 2 % and 4 % (TS) enzyme dose compared to control. In the case of lipase, this translated to an enhanced biogas production as previously observed, but a potential inhibition of the methanogenesis stage, given the low methane content in the biogas (Ma et al., 2015). In the case of cellulase, the increase in hydrolysis rate was marked by an absence of any enhancement of biomethane yield, suggesting that cellulase hydrolyses substrates that are already easily degradable in the digester.

Overall, the study shows the potential to tailor enzymes to matrix macro-composition in the digester. The present study also demonstrated that augmenting the correct type of enzymes can have a complementary and synergistic effect with thermal and biological pre-treatments by targeting the “blind spot” of the AAD in question. While BMP tests enabled the selection of enzymes and optimum dose, long-term validation studies need to be carried out by dosing the shortlisted enzymes (protease for THP-AD and α -amylase for BH-AD) in pilot-scale continuous ADs to confirm the positive impact on biomethane potential as observed in the batch tests and to study potential long term inhibition

and acclimation that cannot be accounted for in batch tests.

4. Conclusion

This study provides new insights into the role of enzymatic pre-treatment in anaerobic digestion, revealing how the effects of enzymes are influenced by the type of feed pre-treatment. Protease increased biogas yield by 16–33 % in THP-AD but had no effect on BH-AD, while α -amylase boosted methane yield by over 30 % for BH-AD. THP pre-treatment improved protein and carbohydrate solubilisation, with proteins partially hydrolysed and carbohydrates fully degraded. Conversely, BH pre-treatment enhanced protein hydrolysis but hindered carbohydrate solubilisation due to EPS accumulation. The study identifies “hydrolysis blind spots” in AD systems and suggests that enzyme addition can optimise biogas production. Overall, this study lays the groundwork for strategic optimisation in the industry by highlighting the need of elucidating the complex interactions between feed pre-treatment, enzymatic activity, and macromolecular solubilisation. Further research on long-term enzyme dosing effects in continuous pilot-scale systems is necessary for industry-wide AD technology improvements.

CRediT authorship contribution statement

Nasreen Nasar: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giulia Pizzagalli:** Writing – review & editing, Methodology, Funding acquisition. **Frederic Coulon:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Yadira Bajón-Fernández:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

All relevant data are provided within the manuscript.

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