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Effects of thermal and enzymatic pre-treatments on the solubilisation of extracellular polymeric substances (EPS) and subsequent anaerobic digestion of microalgae-bacterial biomass

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ABSTRACT

Microalgae-bacteria biomass was digested through anaerobic digestion (AD) to produce biogas. Since microalgae typically have a more resistant cell wall than activated sludge (AS), its anaerobic biodegradability is limited. Therefore, three different pre-treatment techniques were evaluated for their effect on anaerobic biodegradability, especially of the extracellular polymeric substances (EPS). Firstly, heat at 70 °C for 1.5 h. Secondly, exposure to a mixture of commercially available enzymes (cellulase, α-amylase and protease), and thirdly, adding crude hydrolytic enzymes extracted by ultrasonication from a highly loaded anaerobic digestion cascade reactor. The first and third treatments had better effects than enzymatic pre-treatment with commercial enzymes, improving anaerobic biodegradability, the solubilisation of organic matter and increasing the methane production rate by 78 and 21 %, respectively. The EPS content of microalgae-bacteria biomass was considerably lower than reported for WAS, and about 40–50 % of the EPS consisted of proteins and polysaccharides. The hydrolysis of proteins and polysaccharides was quantified, and its effect on AD was discussed. A COD balance showed that the increase of soluble COD is due to the conversion of tightly bound EPS into loosely bound and soluble EPS but also due to the release of organic matter from cellular material. Although all pre-treatments increased the soluble organic fractions, especially those corresponding to EPS, none significantly improved the overall methane yield. Nevertheless, the methane production rate increased after thermal and pre-treatment with hydrolytic enzymes, which could result in smaller and more efficient anaerobic reactors.

1. Introduction

The use of microalgae-bacteria consortia for wastewater treatment (WWT) has been studied for several years as a strategy for organic matter and nutrient removal, potentially reducing operational costs related to aeration and allowing opportunities for resource recovery [1,2]. The waste biomass generated from these systems can be valorised for bio-product production and bioenergy recovery [3]. Previous studies using microalgae-bacteria biomass in anaerobic digestion (AD) have shown promising potential for efficient bioenergy recovery [4]. Moreover, even a positive energy balance can be achieved if AD processes are combined with microalgae-bacteria consortia systems [5].

The AD process entails the removal of biodegradable organic compounds from biomass by converting them into methane and carbon dioxide (CH_4/CO_2) through biochemical steps [6]. Hydrolysis is the first and most essential step in AD, where complex substances are transformed into simple monomeric compounds, which are further converted into the end products [7]. During hydrolysis, extracellular and/or membrane-bound enzymes from hydrolytic bacteria convert complex organic polymers such as carbohydrates, fats, and proteins into soluble monomers like monosaccharides, fatty acids, and amino acids, respectively [8]. However, microalgae are known to have a resistant cell wall even more than the cell wall of activated sludge (AS) bacteria. Therefore, the rate of hydrolysis of microalgae limits the conversion rates in the hydrolysis step and, in addition to that, the efficacy of microalgae-bacteria biomass digestibility and its conversion into methane. The low biodegradability and required high retention times when anaerobically treating microalgae-bacteria biomass results in higher volume

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requirements or long treatment periods affecting construction and operation costs, thus restricting the number of full-scale applications [9,10].

A wide range of pre-treatment techniques are applied preceding AD to increase the hydrolysis rate, as well as the rate and extent of biomass bioconversion [11]. For instance, earlier research concluded that thermal pre-treatment at low or mild temperatures and/or enzymatic pre-treatment using hydrolytic enzymes resulted in effective organic matter solubilisation and, therefore, in improved methane production and positive energy balances of the entire system [12,13]. Low energy requirements and the easy operation characterise the mentioned pre-treatment techniques [11].

Besides the intracellular polymer substances (IPS) of interest, such as proteins and carbohydrates, which are the primary components [14], microalgae-bacteria, like other microorganisms, can also excrete different amounts of extracellular polymeric substances (EPS), which depends on growth conditions [15,16]. In general, EPS are a crucial carbon source in the food chain, which consists of a mixture of complex biopolymers, where carbohydrates and protein (enzymes and structural protein) are also the predominant compounds found during extractions [17,18]. EPS are structural components in matrices of microbial consortia and can be considered external energy storage polymers, providing a heterotrophic carbon source in the microbial food chain. Previous analysis of EPS in microalgal culture and waste activated sludge (WAS) indicated that *Chlorella*, in addition to its more resistant cell wall, also secreted EPS into the surrounding liquid than formed floc-associated EPS as in AS flocs [19,20]. The latter could indicate a more complex or arduous outer structure for microalgae, making it even harder to digest than WAS.

A previous study reports on the effects of the above mentioned pre-treatments on the EPS content in WAS [11]. When low-temperature thermal pre-treatment (55–100 °C) was applied to WAS, the main effects were the disruption of cell membranes (and subsequent release of intracellular contents) and concomitant solubilisation of organic compounds (carbohydrates and protein) present in the EPS [21]. The observed solubilisation could increase the biodegradation rate during subsequent AD and make biomass more susceptible to biodegradation [9,22]. It is hypothesized that the release of more easily degradable organic matter from EPS in the medium and a decrease in particle size after thermal pre-treatment could increase hydrolysis rate during AD [23].

Similarly, when the biological treatments were applied, the addition of enzyme-rich solutions and the in-situ bio-augmentation of enzymes also increased the solubility of compounds and provoked the EPS to detach from the attached cell surface [24,25]. This further increases the hydrolytic enzyme activity due to the liberation of enzymes trapped within the floc matrix [26]. Hence, enzymes acted across the EPS, resulting in sludge disintegration and a decrease in the average particle size [27]. In previous studies, endogenous and commercial enzymes applied to WAS increased soluble organic matter content, mainly consisting of carbohydrates and proteins derived from EPS and lysed cells [28]. Besides, although the application of proteases and enzymatic pre-treatment only resulted in limited solubilisation of proteins and carbohydrates, concomitant increased solubilisation of humic substances was observed, as well as an increased hydrolysis rate [29,30].

Despite the considerable amount of research on WAS pre-treatment, no relevant information was found about the effects of these pre-treatments on EPS composition and distribution in microalgae-bacteria consortia. Regarding the EPS characteristics, results of various researchers indicated a remarkable variation in composition, possibly due to the different growth conditions, used species, age of the culture, physiology, and different EPS extraction and analytical methods used [16,17,31].

Earlier research of microalgae biomass, which was co-digested with different ratios of WAS, showed that biogas yield and methane production rates improved by 73 % - 79 % compared to the AD of sole microalgae as feed [20]. These results indicate that when algae were co-digested with different amounts of WAS (from 59 to 96 % in mass), but the biogas yield of microalgae improved, and the gas phase was reached quickly [20]. Besides, the co-digestion of algae and WAS could improve the dewaterability compared to the individual digestion of algae and WAS. Therefore, it can be expected that the digestibility of microalgae-bacteria consortia is higher than solely microalgae digestion and can bring symbiotic effects such as improved dewaterability, making this biomass more suitable for bioenergy harvesting [20].

Other studies researched substrate solubilisation during pre-treatment of mixtures of different ratios of WAS and microalgae. Results showed that an increase in organic matter solubilisation not necessarily means a proportional increase in methane production [32]. Therefore, other parameters than the biochemical methane potential (BMP) should be considered to determine the effects of pre-treatment, such as organic matter composition, EPS composition and distribution, presence of soluble microbial products (SMPs) and the methane production rate.

The composition and distribution of EPS are considered important for the digestibility of microalgae-bacteria consortia. Therefore, the objectives of this study were:

a) To investigate the biochemical effects of three pre-treatment techniques on the solubilisation of polysaccharides and proteins in EPS: i) mild temperature thermal pre-treatment, ii) application of commercial enzymes (α–amylase, cellulase, and proteinase), and iii) application of hydrolytic enzymes extracted from an anaerobic cascade system.
b) To determine the BMP of microalgae-bacteria biomass after and before pre-treatments and its relation to EPS solubilisation.

The results will help in practice to select an appropriate method to improve the anaerobic digestion of the microalgae-bacteria biomass and thus make its use in wastewater treatment systems more efficient in terms of energy and resource recovery.

2. Materials and methods

2.1. Microalgae-bacteria biomass

The algae-bacteria biomass was cultivated in two laboratory-scale sequencing batch photo-bioreactors (SBPBRs) of five litres each with a hydraulic retention time (HRT) of two days and a solids retention time (SRT) of ten days. The SBPBRs were built with two 5 L vessels (SCHOTT DURAN), peristaltic pumps to control inlet and outlet flow rates, stirring plates to maintain mixing conditions (when necessary), artificial lamp (lamp HQIBT 400v/D proE40), plastic tubing, pH meter (WTW pH 3310), Dissolved Oxygen (DO) meter (WTW Oxi3310) and control timers to change the cycle conditions of a sequencing batch reactor [33]. The main conditions were monitored and modified to ensure the algae-bacteria biomass kept the same characteristics over the different experimental phases. During the inoculation and adaptation phase, the reactors did not have SRT, to achieve the most significant amount of fresh biomass possible in a short time; once the reactors reached their maximum capacity, the daily mixed liquor removal and biomass production was estimated and collected weekly to manage the SRT. The HRT was also controlled by calibrating the pumps with the control timers to keep constant flow rates in the influent and effluent (see supplementary information).

The algae-bacteria biomass used in this study consisted of five microalgae pure strains, namely *Chlorella* sp., *Scenedesmus quadricauda* sp., *Anabaena variabilis* sp., *Chlorococcus* sp., and *Spirulina* sp. obtained from the IHE Institute for Water Education laboratory; and of a concentrated mixed liquor-suspended solids (MLSS) obtained from an AS system in the municipal wastewater treatment plant (WWTP) Harnaschpolder (Den Hoorn, the Netherlands). The algae-bacteria biomass

was grown using primarily settled influent as the substrate, which was also collected from the same WWTP. The SBPBRs were maintained at constant conditions, i.e., room temperature between 25–30 °C, continuous illumination at 335 μmol/m^{-2}/s^{-1}, continuous agitation at 150 rpm, pH-controlled by acid and base addition between 6 and 8, DO was continuously monitored, and the biomass production was estimated to decide the daily wastage of mixed liquor keeping the same SRT in the reactors. After 50 days of operation, the microalgae-bacteria biomass was collected and concentrated by sedimentation for 30 min. Hereafter, the biomass was stored at 5 °C, characterised physiochemically, and used after five days to ensure the same biomass characteristics for all the pre-treatments evaluated.

2.2. Thermal and enzymatic pre-treatments

Low-temperature thermal pre-treatment was carried out in triplicate, using a water bath at 70 °C, applying 1.5 h of exposure time [34]. In this research, two types of enzymatic pre-treatments were evaluated. The first enzymatic pre-treatment was the addition of crude hydrolytic enzymes extracted by ultrasonication from a novel cascade AD system, which was characterised by high enzymatic activities focused on protease and cellulase as main hydrolytic enzymes [25]. The novel cascade AD system consisted of four stages, three 2.2 L ultra-short solids retention times (SRTs) continuously stirred tank reactors (CSTRs), from where the enzymes were extracted, and one 15.4 L CSTR, the system significantly improved the enzymatic hydrolysis rate and extend in AD of WAS in comparison with conventional CSTR digesters [35]. The second enzymatic pre-treatment was the active addition of a mixture of different obtained commercial enzymes from Sigma-Aldrich (cellulase, α-amylase and protease) with a 1 % dose (*w*/w) [36].

Fig. 1 illustrates the extraction protocol of hydrolytic enzymes protocol adapted from G. Yu et al. [37]. 30 mL of extracted solution as a source of hydrolytic enzymes was added into a 200 mL sludge sample to investigate the effects of the hydrolytic enzymes on algae-bacteria biomass according to methods adopted from previous studies [30]. Both enzymatic pre-treatments were conducted in duplicates at an optimal enzymatic temperature of 37 °C, controlled by a water bath, applying 6 h of reaction time [36]. All pre-treatments were under continuous shaking (150 rpm) in a thermostatic shaker water bath, in 300 mL serum bottles and with a liquid volume of 200 mL. The conditions for optimal enzymatic activity were selected based on the range of the general best values found in previous studies where enzyme mix was studied as pre-treatment to improve microalgae biogas production (temperatures: 37–50 °C, dosage: 1–2 %, incubation time: 10–60 min and exposure time: 6 h) [36,39].

The effects of the different pre-treatments were determined regarding the organic matter, polysaccharides (PSs) and proteins (PNs) solubilisation. These last two macromolecules (PSs and PNs) were selected because both organic polymers are essential for an appropriate AD and are the primary EPS and cell wall components [13]. The solubilisation percentage was calculated using Eq. (1) [22] and accounts for the transfer of particulate fraction to the soluble fraction of biomass during pre-treatment.

$$solubilization\ (\%) = \left[\frac{Soluble\ concentration_{treated\ biomass} - Soluble\ concentration_{untreated\ biomass}}{Total\ concentration_{untreated}}\right] * 100 \quad (1)$$

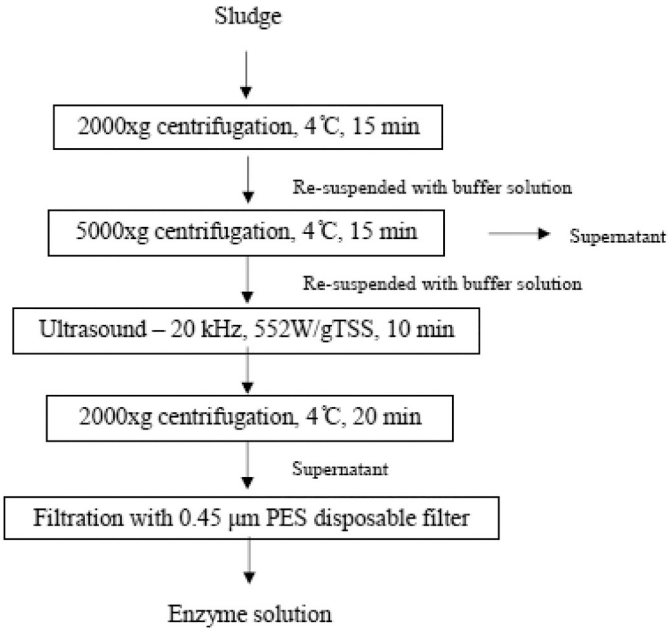

Fig. 1. Hydrolytic enzymes extraction protocol by ultrasonication [38].

2.3. EPS extraction

EPS include metabolites of microbial activity, intracellular materials released by normal cell lysis, and organic matter adsorbed from influent media [37,40]. The applied thermal EPS extraction method was adopted and modified by Morgan et al. [41] for extracting soluble EPS (S-EPS). This fraction may include any other dissolved organic compound not part of the EPS, such as dissolved organic compounds released after cell/death/hydrolysis or even small traces of organic matter from the wastewater used for the growth of microalgae-bacterial biomass. Consecutive thermal extraction will solubilize the loosely bound EPS (LB-EPS), followed by the tightly bound EPS (TB-EPS) surrounding the cells. Fig. 2 illustrates the EPS extraction protocol.

The extraction method consisted of several steps. The first step was centrifugation (4000*g*) for 5 min in 50 mL tubes to dewater the sludge suspension. Then the supernatant or centrate liquor was recovered as the soluble product (S-EPS). The sludge pellet was resuspended into 50 mL of 0.05 % NaCl solution. The NaCl solution was pre-heated to 70 °C to warm the sludge suspension to 50 °C. Then the sludge was mixed in a vortex mixer for 1 min, followed by centrifugation (4000*g*) for 10 min. The extracted supernatant was considered the LB-EPS of the biomass. Lastly, the TB-EPS was extracted by suspending the new pellet again in 0.05 % NaCl solution to 50 mL. This time the sludge mixture was heated to 60 °C for 30 min in a water bath, and then it was centrifuged (4000*g*) for 15 min. The new supernatant corresponded to the TB-EPS [38].

After the extractions of the EPS (LB-EPS, TB-EPS and S-EPS), their composition in terms of PNs, PSs and COD were analysed. After EPS extraction, the samples were filtered using a 0.45 μm membrane filter of cellulose acetate, preserved at −20 °C and further analysed as described in Section 2.4.

2.4. Biochemical methane potential (BMP) tests

Anaerobic biodegradability tests were performed using an automatic methane potential test system (AMPTS II) (Bioprocess Control, Sweden) with 300 mL serum bottles under mesophilic conditions (37 °C) in mixed

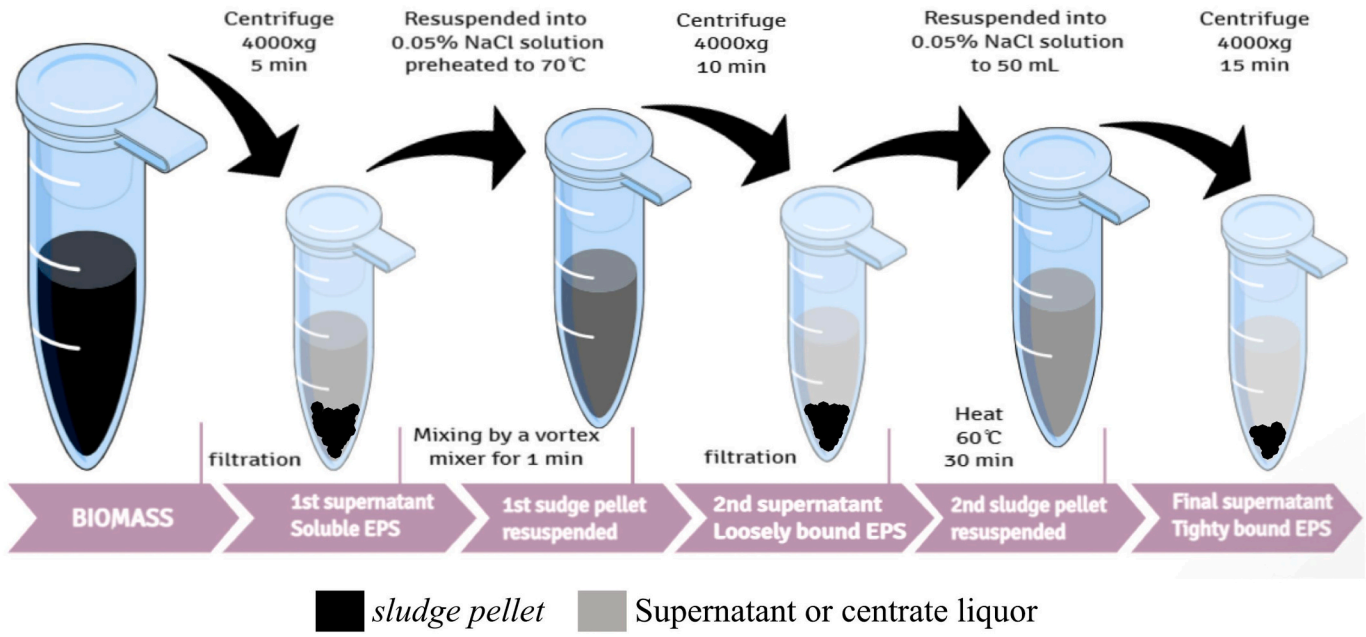

Fig. 2. Schematic representation of the thermal EPS extraction method, adopted and modified from Morgan et al. [41].
EPS released from the sludge after centrifugations or dissolved in the sludge supernatant are referred to as supernatant or soluble EPS.

batches (150 rpm). The active inoculum derived from an anaerobic sludge collected from a full scale digester at the municipal WWTP Harnaschpolder (Den Hoorn, the Netherlands). The substrate-to-inoculum ratio (S/I) was established at 0.5 $gVS_{substrate}/gVS_{inoculum}$ according to previous studies to avoid imbalance due to volatile fatty acids (VFA) accumulation [42,43]. The working volume of 200 mL was calculated accordingly to the S/I ratio selected and considering the physicochemical characterization of the algae-bacteria biomass and the inoculum. All serum bottles had a headspace of about 50 mL. The composition and dosages for the buffer solution, macronutrients, and trace elements were added according to the literature [44,45]. After the substrate and inoculum were added, bottles were purged with a gas mixture composed of nitrogen (80 %) and CO_2 (20 %) to create anaerobic conditions. All tests were performed in duplicates except for the thermal treated biomass performed in triplicates (see Table 2). Produced biogas passed a 97 % NaOH solution for CO_2 stripping. Normalised (at 0 °C, 1 atm and dry gas) accumulated gas production and gas flow rate were calculated by the system (AMPTS II), and the amount of methane produced per gram of VS added was calculated. Results were reported as mean values of the methane yield. For comparing the kinetics effect after pre-treatment conditions, the BMP tests were modelled using a modified version of Gompertz model using Eq. (2) [46].

$$B(t) = P \times exp\left(-exp\left(\frac{Rm \times e}{P}(\lambda - t) + 1\right)\right) \quad (2)$$

where B(t) represents the methane accumulated in time t (mL CH_4/gVS); P represents the maximum potential of methane production (mL CH_4/gVS); Rm represents the maximum rate of methane production (mL CH_4/gVS.d); λ represents the duration of the lag phase (d), and t represents the digestion time (d).

2.5. Analytical methods

Physicochemical parameters, i.e., total solids (TS), chemical oxidation demand (COD), volatile fatty acids (VFAs), total nitrogen (TN) and ammonia (NH_4-N) were determined as indicated in the Standard Methods for Examination of Water and Wastewater, 20th Edition, American Public Health Association, Washington DC [47]. The biomass and EPS extractions were analysed in terms of COD, PNs and PSs before and after pre-treatment to analyse the biomass solubilisation percentages. The effect of each pre-treatment on the organic matter present as EPS and cell wall, PSs and PNs present in the EPS extractions were evaluated. The PNs and PSs were analysed by UV/VIS spectrophotometer following the modified Lowry method using bovine serum albumin as the standard (BSA-Sigma) and the Dubois method using glucose (GLU) as the standard, respectively [48,49]. The final PSs and PNs concentrations were obtained in mgGLU/L and mgBSA/L. However, to analyse the effects of the different pre-treatments in terms of organic matter present as PNs and PSs, the concentrations were converted to COD per gram of VS (mgCOD/gVS).

2.6. Statistical analysis

ANOVA test and *t*-Test were used to analyse variance and compare means to analyse the effects of the pre-treatments on the organic matter solubilisation, EPS content distribution and methane production rate and yield. The pre-treatment type was considered the independent variable, and COD, PSs, PNs, methane production rate, and methane yield were the dependent variables. Differences were considered significant at ρ – values below 0.05, assuming a 95 % confidence level. The Gompertz equation was used to fit the AD data.

3. Results

3.1. Organic matter solubilisation

All pre-treatments raised the COD content in the soluble phase compared to the untreated algae-bacteria biomass (Table 1). However, the highest value was observed for thermal pre-treatment with 11.6 % biomass solubilisation (5.5-fold increase), followed by the enzymatic pre-treatment with crude enzymes with 10.3 % biomass solubilisation (5-fold increase). Lastly, the enzymatic pre-treatment with commercial enzymes showed the lowest solubilisation value, with a 4.2 % (2.5-fold increase) (Table 3).

Results indicate that adding hydrolytic enzymes had a solid ability to hydrolyse microalgae-bacteria biomass. Apparently, the enzyme cocktail extracted from the high-loaded WAS fermenting bioreactors is also

Table 1
Different pre-treatments employed.

Parameters	Pretreated biomass		
	Low-temperature thermal	Hydrolytic enzymes	Commercial enzymes
Conditions	70 °C (water bath)	30 mL enzyme solution in 200 mL algae-bacteria biomass. 37 °C (water bath)	1 %(w/w) dose 37 °C (water bath)
Enzymes	N/A	Cellulase and protease as the main hydrolytic enzymes in the extracted solution	Commercial enzymes from Sigma-Aldrich: o Cellulase from *Aspergillus niger* (≈ 0.8 U/mg) o α – amylase from *Bacillus* sp. (50 U/m) o Proteinase from *Bacillus Licheniformis* (≥ 2.4 U/g)
Exposure time	1.5 h	6 h	6 h
Reference	[34]	[37]	[36]

Control: algae-bacteria biomass without pre-treatment.

Table 2
Anaerobic digestion sets.

Digestion set	Content
Negative control (−) triplicates	Active inoculum, no substrate, and buffer solution with basic medium.
Positive control (+) triplicates	Active inoculum, cellulose as substrate and buffer solution with basic medium.
Untreated duplicates	Active inoculum, untreated biomass as substrate and buffer solution with basic medium.
Thermal triplicates	Active inoculum, thermally treated biomass as substrate and buffer solution with basic medium.
Hydrolytic enzymes duplicates	Active inoculum, biomass treated with hydrolytic enzymes as substrate and buffer solution with basic medium.
Commercial enzymes duplicates	Anaerobic sludge Anaerobic sludge (inoculum), biomass treated with commercial enzymes as substrate and buffer solution with basic medium.

effective in enhancing the solubilisation of microalgae-bacteria consortia [25]. Hydrolytic enzymes and thermal pre-treatment had a more favourable effect on organic matter solubilisation and monomer formation than commercial enzymes.

The solubilisation caused by the pre-treatments was not expected to affect the TCOD in each bottle because of the absence of oxygen or other electron acceptors in significant quantities. The observed values of TCOD and TS were, on average, 8.1 g/L and 8.3 g/L, respectively. These values were not significantly different ($p > 0.05$). Therefore, the results indicated that all pre-treatments caused a disintegration/hydrolysis process, resulting in the yield of soluble organics.

The initial total VFAs levels were very low in the untreated and thermally treated biomass, with acetic acid as the main component. Both enzymatic pre-treatments caused an increase in VFA values, but these values represent not more than 13 % of the SCOD. The increase in enzymatic activity and the ease in accessibility of substrates such as PNs and PSs can lead to better hydrolysis by fermentative microbes resulting in higher VFA yields and consequently improving the biodegradability of the sludge [11,50].

3.2. Effect of solubilisation on EPS fractions

The total EPS in the untreated microalgae-bacteria biomass was equal to 166 mgCOD/gVS (Fig. 3), which is 11 % of the Total COD measured for the untreated biomass (1459 mgCOD/gVS). In the treated biomass, the COD equivalent of the EPS had increased to 16 % for thermal treatment, 18 % for hydrolytic enzymes treatment and 15 % for commercial enzymes treatment. A COD mass balance for the effect of thermal pre-treatment shows that TB-EPS is transformed into LB or S-EPS. The increase in total EPS by the pre-treatment indicates that organic matter was released from cellular material (cell wall or cytoplasm components). After thermal pre-treatment, around 26 % of total EPS consisted of new organics released by cell wall lysis or the release of cellular material (Fig. 3a). The remaining 74 % was EPS that was already present before the pre-treatment. After the two enzymatic pre-treatments, the remaining fraction of TB-EPS was significantly higher than after the thermal pre-treatment, between 35 and 50 % (Fig. 3b-c).

During the solubilisation analysis it was observed that microalgae-bacteria biomass contained significantly less EPS than WAS in previous studies. The solid phase of WAS consists of 50–90 % of EPS, corresponding to EPS and water held within the EPS structure. Assuming a theoretical ratio of 0.8 gVSS/gTSS and 1.42 gCOD/gVSS in the WAS, it gives a fraction of 40–80 % of EPS to the total COD content [51,52].

The soluble EPS-COD values (Fig. 3) in each pre-treatment correspond well with the soluble COD values reported in Table 1, with around 0.2 gCOD/L in the untreated biomass, 1.1 gCOD/L in the thermally treated biomass, 0.5 gCOD/L when commercial enzymes were used and 0.8 gCOD/L when hydrolytic enzymes were used. This indicates a concordance of EPS results with the initial soluble organic matter fraction and confirms the pre-treatments stimulation in the degradation of EPS and cellular material.

Table 3
Characterization of algae-bacteria biomass before and after pre-treatments. Mean values ± SD ($n = 3$).

Parameters	Untreated biomass	Pretreated biomass		
		Thermal	Hydrolytic enzymes	Commercial enzymes
Conditions	–	70 °C, 1.5 h	30 mL enzyme solution 6 h	1 % dose, 6 h Enzymes mix
TCOD (g/L)	8.1 ± 0.3	8.2 ± 0.6[a]	8.2 ± 0.5[a]	8.0 ± 0.1[a]
SCOD (g/L)	0.16 ± 0.05	1.1 ± 0.03[b]	1.0 ± 0.02[b]	0.5 ± 0.01[b]
COD solubilisation (%)	0	11.6	10.3	4.2
TS (g/L)	8.1 ± 0.6	8.3 ± 0.2[a]	9.1 ± 0.4[a]	7.7 ± 0.7[a]
Soluble TN (mg/L)	77.8 ± 2.0	55.1 ± 4.9[b]	38.9 ± 0.6[b]	36.4 ± 2.2[b]
$NH4^+$-N (mg/L)	76.4 ± 2.6	40.6 ± 6.7[b] 76.4 ± 2.6[b]	22.9 ± 2.5[b]	22.7 ± 7.9[b]
pH	7 ± 0.02	6.7 ± 0.1	6.9	6.7 ± 0.1
VFAs (mgCOD/L)	5.5	5.6	48.7	53.2

[a] Stand for values equal to untreated biomass as a control ($\rho > 0.05$).
[b] Stand for significantly different values in comparison with untreated biomass as a control ($\rho \leq 0.05$).

3.3. Polysaccharides and proteins distribution in EPS extractions

In most microalgae, PSs are present in anti-parallel chains, forming cellulose, hemicellulose and pectin, which are PSs consisting of linear chain units. Their detailed structure and abundance vary between species and cell types. Another microalgae cell wall component group is the arabinogalactan proteins (AGPS), which are highly glycosylated proteins (glycoproteins) [53]. The most important biological role of these

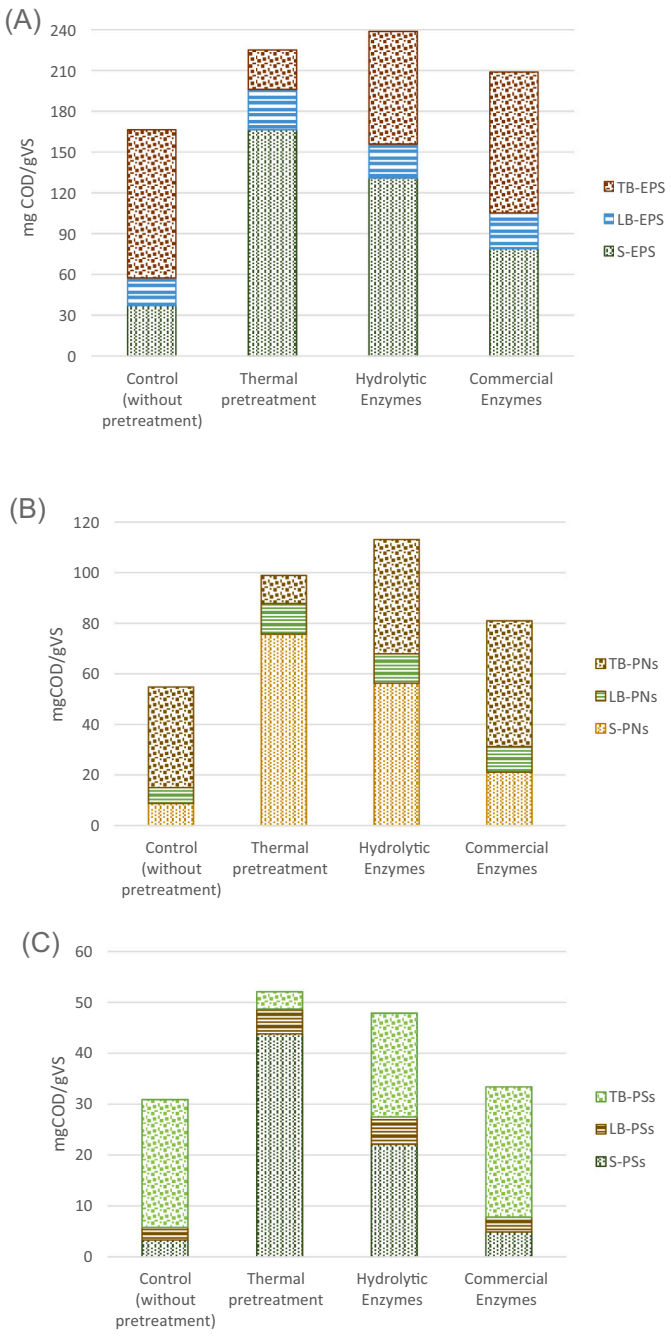

Fig. 3. (A) COD distribution on EPS extractions (total COD in biomass: 1459 mgCOD/gVS for untreated, 1433 mgCOD/gVS for thermal pretreatment, 1302 mg COD/gVS for hidrolytic enzymes, and 1404 mgCOD/gVS for commercial enzymes).
(B) Proteins (PNs) distribution on EPS extractions.
(C) Polysaccharides (PSs) distribution on EPS extractions.
All pretreatment present significant differences in comparison with untreated biomass as a control ($\rho = 0.05$).

PSs and PNs is their contribution to strengthening the cell wall, cell-cell interactions and a structural role in the cell wall, hampering hydrolysis of microalgae [54].

PSs and the PNs are major organic macro components of the cell wall and EPS, with a typical double lipid layer forming cell membranes. PSs and PNs solubilisation are important for efficient anaerobic digestion of the microalgae-bacteria biomass. The fraction of PNs and PSs of the total EPS in all the samples ranged from 26 % to 30 % and 15 % to 22 %, respectively. The sum of PNs and PSs in EPS of microalgae and WAS may even reach 70–80 %. At the same time, the remaining fraction of uncharacterized organic carbon of EPS is possibly composed of humic compounds, nucleic acids and lipids, also as cell membrane components [55].

Solubilisation of PNs and PSs occurred in all pre-treatments but was highest in thermal pre-treatment, which it increased 8.6 and 13.3 fold, respectively. In hydrolytic enzymes pre-treatment, these figures were 6.7 and 6.4 fold; for commercial enzymes treatment, only 2.4 and 1.5 fold, respectively. The increase in total PN and PS (based on COD) in the EPS layers cannot be fully explained by the solubilisation of the EPS of the untreated biomass (Fig. 3b and c). Therefore cell wall lysis of cell disruption could also have contributed to PN and PS release.

3.4. Methane production in BMP test

Thermal pre-treatment did not cause an increase in the accumulated methane yield if compared with untreated biomass, even though the thermally treated biomass was shown to have a more significant fraction of sCOD and S-EPS, including PSs and PNs. However, the organic matter solubilisation by thermal pre-treatment favoured the degradation rate (Rm), increasing it considerably from around 32 mL CH_4/gVS.d to around 57 mL CH_4/gVS.d (78 % increase) (Table 4).

For the hydrolytic enzymes' treatment, the average final methane yield increased by 12 % compared with the untreated biomass from 113.3 mLCH4/gVS to 127.2 mLCH4/gVS, but this difference was not statistically significant. However, after enzymatic pre-treatment with hydrolytic enzymes, the degradation rate increased significantly, 21 % from 31.8 mLCH_4/gVS.d to 38.8 mLCH_4/gVS.d. For the commercial enzyme treatment, no significant differences were observed in the final methane yield and the degradation rate.

The COD of the untreated biomass (1492 mgCOD/gVS) was only 23 % converted into CH_4-COD, which represented about double the COD of the EPS fraction. This implies that during AD of the untreated biomass, part of the cell wall/cytoplasm is digested, not only the EPS. Similar percentages were obtained for AD of thermal pre-treatment and commercial enzymes treatment, i.e., 22 % and 25 %, respectively.

In contrast, the pre-treatment with hydrolytic enzymes also increased the average methane production (P) to around 363 mgCOD/gVS, which represents approximately 28 % of total COD in the biomass content and an increase of 13 % in the final methane yield in comparison with the digestion of the untreated biomass. However, even though adding hydrolytic enzymes increased the soluble organic matter by

Table 4
Experimental data and data collected from the modified Gompertz model used in the BMP test. ʎ: latency period; R_m: rate of biogas production (mL CH_4/gVS.d); P: maximum biogas production (mL CH_4/gVS); R^2: coefficient of determination.

Pretreatment	ʎ (day)	Rm (mLCH4/gVS.d)	P (mL CH4/gVS)	P (mg CH_4-COD/gVS)	R^2
Untreated					
AB1	0	33.2	117.7	335.4	0.98
AB2	0	30.4	108.8	310.1	0.94
Thermal					
T1	0	54.8[a]	114.1	325.2	0.99
T2	0	53.2[a]	106.1	302.4	0.99
T3	0.02	62.3[a]	110.1	313.8	0.98
Hydrolytic enzymes					
N1	0	39.2[a]	139.4	397.3	0.96
N2	0	38.3[a]	115.1	328.0	0.96
Commercial enzymes					
C1	0	33.5	118.8	338.6	0.95
C2	0	31.2	123.6	350.6	0.96

[a] Stand for significant differences in comparison with untreated biomass as a control ($\rho < 0.05$).

about 75 mgCOD/gVS, P only increased by about 41 mgCOD/gVS without a statistically significant difference. This confirms previous studies where there is disproportionality between the solubilisation of the biomass mixture (sCOD) and methane production effects [32]. Therefore, the increase in solubility does not directly translate into a higher P. Nonetheless, it could mean an increase in methane production (Rm) rate during AD of algae-bacteria biomass.

Results for pH, VFAs and ammonia before AD show that the initial levels were within the optimal range (pH 6.5–8.0 and ammonia nitrogen below 1.7 g/L) to obtain a maximum methane yield. As mentioned before, the total VFAs levels for the untreated and thermally treated biomass before AD were already low. However, after enzymatic pre-treatments, the VFAs values increased; although these values were not representative (not more than 13 % of the SCOD), they decreased considerably after AD in comparison with the initial concentrations. On the contrary, ammonia concentrations increased substantially after AD in all the samples (Table 5).

4. Discussion

4.1. Pre-treatment effects that promote organic matter solubilisation

All the pre-treatments increased the soluble organic matter fraction due to the degradation of macromolecules present in the microalgae-bacteria biomass. The effect of low-temperature heating and the addition of hydrolytic enzymes was more pronounced than the effect of the addition of commercial enzymes. These macro-molecules originated from EPS, cell walls and other cellular components. Particle fractions present during pre-treatments in the microalgae-bacteria biomass were hydrolysed, transforming complex organic matter and macro components into soluble fractions and less complex compounds [56,57]. Solubilisation of particulate matter is considered crucial for efficient anaerobic digestion [13].

The results obtained for biomass solubilisation during thermal pre-treatment are in accordance with previous studies at mild temperatures for microalgae and WAS, where the soluble COD concentration increased considerably by 4 fold and 3 fold, respectively, in comparison with untreated biomass [22,58,59]. Applying mild temperatures is beneficial at an industrial scale due to its easy operation, no need for pressurized vessels, and (almost) absence of formation of recalcitrant compounds during pre-treatment compared with high temperatures [60].

The pre-treatment with commercial enzymes obtained the lowest organic matter solubilisation in this study. However, previous analyses showed similar effects when a 1 % dose of commercial enzyme mix was applied during 6 h to a mixed microalgae biomass (similar conditions), with a 3.4-fold increase in soluble organic matter [36,39]. Also, another study where a nonspecific protease dry powder (SIGMA product) was tested to see the effects on chemical and physical properties of WAS, showed a total 2.9-fold increase in the soluble COD content after pre-treatment [27]. Protease addition in microalgae also resulted effective for the highest organic matter solubilisation and, therefore, the highest methane production [61]. Nonetheless, it is recommended that to have better saccharification efficiency, different enzymatic hydrolysis factors must be optimised for the specific algae-bacteria biomass studied to result in better organic matter solubilisation and, therefore, higher methane yield. For instance, enzymes ratios, enzyme dosages, temperature, incubation period and exposure time to the release of remarkable monosaccharide concentrations. The higher enzymatic dosage could increase the release of reducing sugars as more enzymes are available for action on the substrate [62]. The low efficiency of the commercial enzymes due to the low optimization of the enzymatic conditions, could have resulted in a low yield of soluble organic matter compared to the effects of the hydrolytic enzymes.

A strategy found in previous studies to overcome microalgae cell wall resistance is bacterial bio-augmentation and adding bacterial cultures from different low-cost substrates, such as WAS and sludge from anaerobic digestion systems. These low-cost substrates contain a variety of active microorganisms in different quantities, providing a constant source of in-situ endogenous enzymes [28,63,64]. Such bio-augmentation could result in continuous hydrolysis of sludge by residual endogenous enzymes during pre-treatment. The results from the present study were similar to previous research in which the effects of amylase and protease extracted from WAS were studied to improve the efficiency of anaerobic sludge digestion, showing values from 7.5 to 17.1 % organic matter solubilisation [30,64]. The extraction of hydrolytic enzymes from fermentation broth could potentially reduce the cost of enzymatic pre-treatment since purchasing commercial enzymes adds to the operational costs.

In the end, resulting compounds present in the soluble fraction after pre-treatment can be assimilated more easily by microorganisms, consequently improving and facilitating methane production. As expected, the additional degraded organic matter could come from EPS and cellular material, mainly translated into PSs and PNs available in the soluble fraction for AD [20,65].

4.2. Solubility of polysaccharides and proteins

Previous research in WAS digestion obtained similar results when the low temperature was applied, with a soluble PSs concentration increase from 5.3 to 8 fold and a soluble PNs concentration increase from 9 to 25 fold compared with the control [22]. Other studies also show that adding hydrolytic enzymes enhanced the solubilisation of PSs and PNs when carbohydrases and proteases were applied to *Chlorella vulgaris* and *Scenedesmus* sp. for microalgae hydrolysis prior to AD [28]. Their results show high organic matter solubilisation (47 %) with specifically high carbohydrate solubilisation (84 % for *C. vulgaris* and 36 % for *Scenedesmus sp*). Ultimately, the effect of the pre-treatments, i.e., increased solubilisation of PSs and PNs, increased biodegradation and thus, increased methane production rate [11,78].

Similar to other studies related to WAS conversion, we observed that solubilisation of both components did not exceed 20 % of the total organic fraction of the microalgae-bacteria biomass, meaning a significant amount of PNs, PSs and other organic compounds could remain bound in the matrix during pre-treatments. Based on our results, it can be inferred that moderate temperatures and/or biological pre-treatment with hydrolytic enzymes resulted in sufficiently better monomer solubilisation than the addition of commercial enzymes and thus improved digestibility and methane production performance.

4.3. EPS in microalgae bacteria consortium

Microalgae-bacteria biomass was found to have a lower fraction of EPS (measured in COD) as compared to WAS only, even though the percentage of EPS increased from 11 % in the untreated biomass to 15–18 % in the pretreated biomass. In comparison with WAS, the microalgae-bacteria biomass seems to have significantly less EPS content since the total mass of EPS (EPS and water held within the EPS structure) has been found to represent up to 80 % of the mass in WAS [11,66]. EPS in WAS also may constitute 50–60 % of the total organic matter, while cell biomass only contributes up to 20 % [67,80]. Our results confirm that the microalgae-bacteria biomass is distinctly different from WAS with regard to EPS content. In microalgae-bacteria,

Table 5
Measurements before and after anaerobic digestion.

Pretreatment	pH		VFAs (mg/L)		Ammonia – NH_4 (mg/L)	
	Before	After	Before	After	Before	After
Untreated	7	8.3	5.5	4.5	76.4	90.6
Thermal	6.7	8.1	5.6	0	40.6	81.5
Hydrolytic Enzymes	6.9	8	48.7	5.4	22.9	186.3
Commercial enzymes	6.7	8	53.2	3.4	22.7	84.5

less organic matter is attached to the sludge as TB-EPS, while during pre-treatment, it releases smaller quantities of EPS into the growth medium. Therefore, it may be expected that microalgae-bacteria biomass will generate less biogas during AD compared to the digestion of WAS.

The lower EPS fraction in microalgae-bacteria biomass, compared to WAS, is due to the low EPS excretion by the microalgae. This is in line with the observation that bio flocculation of microalgae is very limited due to the negative cell surface and the small size of most microalgae [68]. Therefore, harvesting microalgae cultures by sedimentation is hampered but can be improved by growing microalgae-bacteria biomass that forms more stable aggregates due to better bio flocculation [79]. Its morphology is like WAS flocs in the sense that the aggregation of microalgae and bacteria make them larger in size, while the consortium is more stable and is characterised by a high settleability. The presence of more stable flocs permits a simple separation of the biomass by gravity sedimentation, reducing the harvesting cost in the system.

Mixing microalgae and bacteria could also be advantageous for anaerobic digestion. Co-digestion of microalgae biomass with WAS improved not only methane yields but also the rate of digestion in a previous study [20]. This can be explained by the higher EPS fraction in WAS as compared to microalgae alone and by the presence of some hydrolytic bacteria in WAS. Our results showed that approximately 40–50 % of the organic matter in the microalgae-bacteria EPS, including S-EPS, LB-EPS and TB-EPS, consisted of PNs and PSs. The thermal and enzymatic pre-treatments caused the EPS to detach from the cell surface more efficiently and further disrupted the EPS matrix, resulting in enhanced solubilisation of complex organic matter, mainly PNs and PSs [69].

As mentioned already, the hydrolytic enzymes for the pre-treatment of microalgae-bacteria biomass result in cost advantages in the system. In addition, such as recent studies in WAS, a cascade anaerobic digestion system could also be considered for the microalgae-bacteria AD, consisting of small volume reactors in series to digest microalgae-bacteria biomass instead of the conventional continuous stirred tank reactors (CSTR) to analyse the reduction of the TCOD during the pre-treatment and digestion [25]. It could result in better performance and acceleration of the hydrolysis rate, improving methane production without the necessity of extracting the hydrolytic enzymes, which can also be costly.

4.4. *Effect of the pretreatments on the methane production*

The thermal pre-treatment and the addition of hydrolytic enzymes improved the methane production rate (Rm) significantly. Previous studies obtained similar results [70,71] when low-temperature thermal pre-treatments and enzymatic pretreatment were applied to microalgae biomass and WAS separately. These studies showed no significant increase in the final methane yield, but the anaerobic degradation rate was up to 70 % higher for pre-treated biomass [30,72,73].

The addition of hydrolytic enzymes resulted not only in the improvement of the methane production rate but also increased the average methane yield (13 %), although the statistical analysis could not confirm that this increment was significantly different from the untreated biomass. In the literature was found that AD of microalgae biomass reached a higher methane yield after enzymatic pre-treatment, with a 15 % increase in accumulated biogas production [36]. Adding endogenous hydrolytic enzymes to anaerobically digested sludge represented a 20 % increase in biogas production [30]. Indeed, some information is still missing about the optimum conditions required for adding hydrolytic enzymes, and its effect can vary depending on the type of biomass and its growing conditions. For instance, it may have some drawbacks, such as low purity of enzymes or culturing proper microorganisms with the correct conditions in the case of bio-augmentation with demands of pH control and substrate addition [11,74]. Enzymatic pre-treatment of microalgae-bacteria biomass with hydrolytic enzymes has the advantage of lower energy requirements compared with the thermal pre-treatment, although using of low-quality heat is a potentially inexpensive way to increase microalgae-bacteria biomass biodegradability. The optimal conditions required for using enzymatic pre-treatment of microalgae-bacteria biomass with hydrolytic enzymes are still to be determined. Follow-up research should investigate the effect of enzymatic pre-treatment using continuous anaerobic digestion systems for producing hydrolytic enzymes to optimize performance, achieve a better energy balance and improve the economics of the process.

As mentioned before, optimal conditions for enzymatic activity with commercial enzymes in algae-bacteria biomass also need to be better investigated since better conditions like enzymes ratio, enzyme dosage, temperature, incubation period and exposure times may improve the concentrations of monosaccharides or soluble organic matter after pre-treatment also resulting in better methane yield from organic matter after digestion [62]. However, other reasons may be linked to these low methane yields from organic matter compared to hydrolytic enzymes. Protease addition in microalgae results in organic matter solubilisation, but it can also inhibit anaerobic digestion due to the release of large amounts of ammonium nitrogen [61]. Possible solutions to overcome this negative effect include reducing protein biomass levels by culturing the microalgae in low-nitrogen media or using ammonia-tolerant anaerobic inoculum. In other cases, protease content can also split glycosidic bonds that link sugar monomers in carbohydrates, resulting in cellulase degradation. This degradation can produce smaller molecules competing with the cellulose and starch substrates for the attention of cellulase and amylase enzymes. Proteases can also directly inhibit the activity of cellulases and amylases through the called enzyme inhibition, and this can occur through different mechanisms: competitive inhibition, where proteases compete with cellulases ad amylases for the same substrate; Non-competitive inhibition, where proteases can bind to the cellulase and amylase enzymes at a location other than active site altering the enzyme's shape and reducing its activity; or by uncompetitive inhibition, where proteases can bind enzyme-substrate complex preventing the substrate from being released and reducing the activity of the enzyme [75]. Therefore, it could have inhibited these enzymes' activity and reduced the reaction's overall efficiency compared to the hydrolytic enzymes' results.

Regarding the insights of the possible mechanisms of the AD process. First, it is observed that in all the cases, the initial conditions (pH, VFAs and ammonia) were optimal to achieve the maximum methane yield in all the scenarios [76]. Although in the samples with enzymatically treated biomass, the decrease in VFAs is more noticeable due to their high initial concentration after enzymatic pre-treatment due to possible hydrolysis by fermentative microbes and, therefore, higher VFAs yields. In all cases, a decrease in VFAs concentrations occurred due to the methanogenesis, where the VFAs are converted in biogas; at the same time, the pH values increased since during consumption of the VFAs in the AD system, a large amount of hydrogen is also consumed during the methane formation. On the other hand, at the end of the AD, a considerable increase in ammonia concentration is observed since the proteins contained in the biomass are also degraded into ammonia during digestion [77]. However, these ammonia concentrations are still below the methanogenesis inhibition effects (>1.7 g/l) whereby an inhibitory reaction is discarded.

5. Conclusions

The following can be concluded on the effects of thermal and enzymatic pre-treatments of microalgae-bacteria biomass on the solubilisation of EPS and subsequent anaerobic digestion:

- The applied pre-treatment methods (60 min heating to 70 °C, addition of hydrolytic enzymes from a high loaded anaerobic digester, and addition of commercially available proteases and cellulases) resulted in solubilisation of organic matter of algal-bacterial biomass. The percentages of solubilisation were 11.6, 10.3 and 4.2 %, respectively.

- About 40–50 % (on COD basis) of the EPS of microalgae-bacterial biomass (soluble, loosely bound and tightly bound) consisted of proteins and polysaccharides.
- The % EPS (on COD basis) increased from 11 % for untreated biomass to 15–18 % for the pre-treated biomass. This range was considerably lower than reported for WAS (40–80 % on COD basis).
- Solubilisation by the pre-treatments was not only solubilisation of bound-EPS but also release or solubilisation of cellular components, as was proven from the COD balance on EPS fractions.
- Although all pre-treatments did increase the soluble organic fractions, they did not significantly increase the overall methane yield. However, thermal treatment and hydrolytic enzyme additions significantly increased the methane production rate, which could result in smaller and more efficient reactors.

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

Data will be made available on request.

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Appendix A. Supplementary data

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