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IMPACTS OF MICROALGAE PRE-TREATMENTS FOR IMPROVED ANAEROBIC DIGESTION: THERMAL TREATMENT, THERMAL HYDROLYSIS, ULTRASOUND AND ENZYMATIC HYDROLYSIS.

Francesco Ometto[i], *Gerardo Quiroga*[ii], *Pavel Pseničkă*[iii], *Rachel Whitton*[i], *Bruce Jefferson*[i] *and Raffaella Villa*,[i,*].

[i] Cranfield University, Bedfordshire (UK)

[ii] University of Oviedo, Oviedo (ES)

[iii] Czech University of Life Sciences Prague, Prague (CZ)

*corresponding author: r.villa@cranfield.ac.uk

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Abstract

Anaerobic digestion (AD) of microalgae is primarily inhibited by the chemical composition of their cell walls containing biopolymers able to resist bacterial degradation. Adoption of pre-treatments such as thermal, thermal hydrolysis, ultrasound and enzymatic hydrolysis have the potential to remove these inhibitory compounds and enhance biogas yields by degrading the cell wall, and releasing the intracellular algogenic organic matter (AOM). This work investigated the effect of four pre-treatments on three microalgae species, and their impact on the quantity of soluble biomass released in the media and thus on the digestion process yields. The analysis of the composition of the soluble COD released and of the TEM images of the cells showed two main degradation actions associated with the processes: (1) cell wall damage with the release of intracellular AOM (thermal, thermal hydrolysis and ultrasound) and (2) degradation of the cell wall constituents with the release of intracellular AOM and the solubilisation of the cell wall biopolymers (enzymatic hydrolysis). As a result of this, enzymatic hydrolysis showed the greatest biogas yield increments (> 270%) followed by thermal hydrolysis (60 – 100%) and ultrasounds (30 – 60%).

Key words: microalgae pre-treatment; cell wall degradation; anaerobic digestion; energy balance.

1. Introduction

Currently anaerobic digestion (AD) provides the most feasible process for large-scale application of algal biomass-to-energy. AD does not require highly concentrated or pre-dried biomass, which eliminates the energy inputs for feedstock preparation required by other processes (Pragya et al., 2013), and, depending on the chemical composition of the species used, the process could yield up to 800 mlCH_4 gVS^{-1} (Heaven et al., 2011).

However, structure and chemical composition of the cell wall of some microalgae species offer a barrier to microbial degradation (Atkinson et al., 1972; Burczyk et al., 1999), resulting in significantly lower methane yields than expected (Golueke et al., 1957; Ometto et al., 2014). Components such as cellulose and acetolysis resistant biopolymers (ARB) like sporopollenin and algaenan, can influence the yields of bacterial degradation and thus the amount of intracellular algogenic organic matter (AOM) released in the media for methane production.

High energy (thermal and ultrasound) and low energy (mechanical and biological) pre-treatments have been used to degrade the cell wall for releasing AOM and enhancing methane production (Alzate et al., 2012; González-Fernández et al., 2012; Cho et al., 2013a). For example, a mixture of microalgae pre-treated at 110°C, 140°C and 170°C for 15 minutes showed increase in methane yields of 19%, 33% and 46% respectively from a control value of about 270 ml gVS^{-1} (Alzate et al., 2012). Similar increases in methane production were obtained with lower pre-treatment temperatures at 55°C, 75°C and 95°C (11%, 21% and 39% respectively) using longer treatment times (5 hours) (Passos et al., 2013).

When using ultrasound, the required specific energy input depends on the physical characteristics of the algae cell, e.g. shape, size and intracellular structure (Purcell et al., 2013). For example, when the filamentous algae *Microspora* sp. was treated at 57 MJ kg^{-1}, a 60% increase in total COD solubilisation and 22% in methane production was achieved (Alzate et al., 2012). Higher energy inputs were required to process single cell algae such as *Scenedesmus* sp. (130 MJ kg^{-1}) and *Chlorella* sp. (1600 MJ kg^{-1}) to double the methane production from 164 to 306 ml gVS^{-1} and from 250 to 450 ml gVS^{-1}, respectively (González-Fernández et al., 2012; Park et al., 2013).

Very often, however, the additional methane yield is not enough to offset the energy required for the pre-treatment, leading to an overall negative energy balance (Cho et al., 2013a; Passos et al., 2013). Consequently, low energy pre-treatments are expected to achieve a more balanced process. Mechanical pre-treatments, such as quartz sand grinding under wet or dry conditions, have shown limited benefits when used for lipid extraction with *Chlorella* sp. (Zheng et al., 2011). In contrast, the same authors reported successful results with biological pre-treatments using enzymatic additions of cellulases. In agreement to this, Yin *et al*. (2010) observed that the addition of cellulases to *Chlorella sorokiniana* enhanced cell wall degradation and produced the release of 25, 6 and 8 times more proteins, peptides and sugars respectively, after three hours at 50°C. Similarly, the addition of cellulase to *Chlorella vulgaris* to optimise lipids extraction produced 60% and 85% enzymatic hydrolysis yields, after 24 h and 72 h treatment (Cho et al., 2013b).

Only few recent works investigated the effect of enzymatic pre-treatment on AD, reporting significant methane yields increment and up to 90% biomass degradation (Ehimen et al., 2013; Ciudad et al., 2014; Mahdy et al., 2014). For instance, an enzymatic mixture containing protease, α-amylase, xylanase, lipase and cellulase used on *Rhizoclonium* biomass (filamentous green algae) produced 40% methane increment (Ehimen et al., 2013). Similarly,

a commercial multienzymatic mixture containing a carbohydralase and a protease, showed 14% methane production enhancement when used on *C. vulgaris* (Mahdy et al., 2014).

Batch anaerobic digestion experiments were used to assess the effect of four different pre-treatments (thermal, thermal hydrolysis, ultrasound and enzymatic hydrolysis) on the methane production of three photosynthetic microorganisms (*Scenedesmus obliquus*, *Chlorella sorokiniana* and *Arthrospira maxima*) commonly found in wastewater treatments plants. The algae and cyanobacteria, chosen for their differences in cell wall structure and composition, were tested under different operating conditions and optimised for maximum soluble COD release and biogas production. In addition, this paper provides the first insight into the degradation mechanism of physical and biological pre-treatments and on their effects on the structure of the microalgal cell wall.

2. Material and methods

2.1 Analytical methods

The algae biomass was characterised for solids content and soluble matter composition (COD, proteins and carbohydrates) before and after each treatment. COD and solid content were measured in duplicate using standard methods (APHA). Soluble matter was obtained after centrifugation at 60,000 rpm for three minutes and syringe filtration (0.45 µm). The soluble protein (sP) and soluble carbohydrate (sC) contents were quantified using the methods described by Frølund et al. (1995) and Dubois et al. (1956). Protein content was measured at 750 nm as bovine serum albumin (BSA) equivalent (Sigma-Aldrich, UK), while carbohydrate content was measured at 480 nm as glucose equivalent (Sigma-Aldrich, UK). The lipid content, defined as "Other compounds including lipids", was estimated subtracting proteins and carbohydrates sCOD equivalent from the total sCOD, 1.25 gO_2 $gBSA^{-1}$ and 1.07 gO_2 $gGlucose^{-1}$, respectively. A statistical study on the sCOD set of data was performed using a Scheirer-Ray-Hare test with a significant acceptance at a p value equal to 0.05. The ratio

between volatile suspended solids (VSS) and total volatile solids (VS) was adopted as the solubilisation index.

Visual inspection of the algae cells was performed using an optical microscope and TEM analysis. For TEM sample preparation, concentrated cell paste was washed with a series of rinses (sodium cacodylate buffer) and fixatives (Gluaraldehyde, Osmium tetroxide and Uranyl acetate), dehydrated with Ethanol and Propylene oxide and embedded in Araldite CY 212 as described by Audrey et al. (1998). All analyses were carried out in triplicate.

2.2 Algal cultures

All algae cultures were obtained from the Culture Collection for Algae and Protozoa (CCAP), (Oban, UK). *S. obliquus* (276/42) and *C. vulgaris* (211/BK) were grown on Jaworski media at 18°C under constant illumination while *A. maxima* (1475/9) was grown on Zarrouk media at 28°C using a 16/8 hours light/dark cycle (Ometto et al., 2014). The algae biomass was collected during the stationary growth phase and concentrated to 20 ± 2 g l^{-1} as total solids (TS) by a combination of sedimentation and centrifugation. Samples were stored at 4°C, for a maximum of 7 days, before pre-treatment.

2.3 Optimisation of biomass degradation

2.3.1 Thermal and Thermal Hydrolysis pre-treatment

Thermal (T) and thermal hydrolysis (TH) treatments were undertaken using a Baskerville autoclave and steam generator WON15827 (Manchester, UK). The unit is composed of two connected pressure vessels: a reactor vessel and a steam generator. For thermal treatments, an aliquot of concentrated algal biomass (4g in 200 ml) was placed in the reactor vessel, heated and maintained at the required temperature for 30 minutes. For thermal hydrolysis treatments, steam was first generated in the steam generator vessel at the required temperature and then injected into the reactor unit containing the algae biomass, pre-heated at 70ºC.

Five temperatures were tested using both configurations: (1) 105°C, (2) 120°C, (3) 145°C, (4) 155°C and (5) 165°C, with associated saturated pressure close to 1, 2, 3, 5 and 7 bar. The final volume of the sample was measured and used for further calculations.

2.3.2 Ultrasound pre-treatment

Five specific energy inputs (Ei) were applied to each sample using a Hielscher Ultrasound UP400S (Teltow, DE): (U1) 0.35, (U2) 3.5, (U3) 10, (U4) 20 and (U5) 35 MJ kg TS^{-1}. Between 250 and 500 ml of concentrated algae was placed in a glass beaker and then placed in an ice bath to limit ultrasonically derived temperature increases. The power input was fixed at 100W (24 kHz) and the fixed energy input was achieved with a consequential exposition time of 50 sec (U1), 5 min (U2), 8 min (U3), 10 min (U4) and an additional 10 min (U5), respectively. Minor deviations from these values were observed due to equipment sensitivity and different TS in the initial samples according to Equation 1:

$$E_i = \frac{P \times t}{V \times \mathrm{TS}} \quad (1)$$

where P represents the power (Watt), t the exposure time (seconds), V the sample volume (millilitres) and TS the total solid concentration (g l^{-1}). At the end of each treatment time, 100 ml of sample was collected and used for analysis.

2.3.3 Enzymatic pre-treatment

Five commercial enzymes (Table 1), were used alone or in combination at different concentrations, 25, 50, 150, 250, and 350 U ml^{-1}, to identify optimal degradation conditions. Between 5 to 10 ml of algae were centrifuged, re-suspended in 0.1 M phosphate buffer at pH 6 to reach 2% TS, and incubated with the enzymes for 24 h at 50°C. The released soluble content was measured as sCOD as reported previously.

2.4 Batch anaerobic digestion test

Anaerobic digestion batch experiments were performed using a modified method of Angelidaki et al. (2009). Briefly, tests with untreated and treated algae biomass (substrate)

were conducted using a substrate:inoculum ratio of 1:1 volatile solid (VS) ratio, with digested sludge obtained from a local wastewater treatment plant as inoculum. Samples were flushed with N_2 gas, sealed with a PTFE crimp cap, and placed at 38°C under constant agitation (150 rpm). Biogas production was determined every two to three days until no significant gas production was detected, for a maximum of 35 days. Data was converted to standard temperature and pressure (STP). The biogas volume produced by the tested substrates was corrected by subtracting the average blank controls production (inoculum + nutrients). Methane content was detected using a Servomex 1440 gas analyser (Crowborough, UK). For the digestion of enzymatically pre-treated algae, using E1, E2 and a 1:1 (E1:E2) mixture at 150 u ml^{-1}, separate blank tests were carried out to quantify the effect of the enzymatic addition to the inoculum digestion. All experiments were conducted in triplicate and cellulose was used as an external control to verify inoculum activity over time.

3.5 Energy balance

The ratio between energy input and energy output (E_i/E_o) of each pre-treatment condition was considered as an indication of the energy balance. Values lower or equal to 1 represent positive and neutral balance, respectively (Passos et al., 2013). The energy output (E_o = kJ gVS^{-1}) was measured on the net increase methane content (ΔP) expressed in ml CH_4 gVS^{-1}, multiplied by the methane heating value (ξ = 35,800 kJ mCH_4^{-3}) as reported in Equation 2:

$$E_o = \frac{\Delta P \times \xi}{10^6} \qquad (2)$$

The energy input (E_i = kJ gVS^{-1}) was estimated using Equation 1 (2.3.2) for ultrasound treatment, and Equation 3 (Passos et al., 2013) for thermal and enzymatic treatments, where the main energy input was related to the heat required to raise the biomass from the initial temperature (T_0) to the pre-treatment temperature (T_p). T_0 was assumed equal to the ambient temperature (20 °C) while T_p was equal to 50°C, 105°C, 120°C, 145°C, 155°C or 165°C.

Microalgal suspension was assumed equal to water for specific density (ρ) and specific heat (γ) values, 1 g ml^{-1} and 4.18 $x10^{-3}$ kJ g^{-1} °C^{-1}. Heat losses were assumed to be negligible, while heat recovery efficiency (ϕ) was assumed equal to 85%.

$$E_i = \frac{\rho \times V \times \gamma \times \left(T_p - T_o\right) \times (1 - \phi)}{\mathrm{VS}} \qquad (3)$$

3 Results and Discussion

3.1 Optimisation of biomass degradation

3.1.1 Thermal and Thermal Hydrolysis pre-treatment

During both thermal (T) and thermal hydrolysis (TH) pre-treatments, the biomass was subjected to a combined thermal and pressure increase, causing cell degradation and a proportional incremental release of the sCOD component (Figure 1). Preliminary investigations demonstrated that the range of pressures reached during the experiments, from 1 to 7 bar, were alone too low to affect the microalgae cell structure (data not shown). It was therefore assumed that the temperature was the main degradation mechanism (Valo et al., 2004; Cho et al., 2013a).

After thermal hydrolysis (TH), the amount of sCOD released by *S. obliquus* and *C. sorokiniana* increased with the temperature to a maximum of 508 mg gTS^{-1} (22 fold) and 400 mg gTS^{-1} (5.4 fold), respectively (raw biomass characteristics available in Table 2) . This was translated in a VSS/VS ratio decrease, from an initial value close to 1, to 0.58 ± 0.02 with both algae corresponding to a 40% biomass solubilisation (Table 3). Similarly, Cho et al. (2013a) obtained a 5.5 fold sCOD increase after autoclaving a mixture of *Scenedesmus* sp. and *Chlorella* sp. for 30 min at 120°C, while Keymer et al. (2013) obtained a 10 fold sCOD increase at 170°C using a mixture of natural algae, confirming comparable treatment efficiency. In contrast, the cyanobacteria *A. maxima* released a constant amount of sCOD between 105°C and 155°C with a VSS/VS ratio between 0.71 and 0.78 across the range. At

165°C the sCOD concentration doubled to 116 mg gTS^{-1} in both pre-treatments (Figure 1), reaching a 65% solubilisation, higher than the one obtained with the single cell algae.

At temperatures lower than 150°C, each algal species released similar amounts of sCOD with the two pre-treatments, confirming our initial hypothesis that identified temperature as the main degradation mechanisms. At temperatures higher than 150°C, the two green algae released significantly more sCOD with the TH (steam injection) treatment, whereas the sCOD increase with the filamentous cyanobacteria was again similar for both pre-treatments. This suggests that, for single cell algae characterised by the presence of carbohydrates polymers/matrix and ARB, the rapid change of temperature/pressure caused by steam injection was only effective at pressures and temperatures higher than 4 bar and 150°C respectively, while for cellulose free filamentous algae, lower temperature/pressure combinations were sufficient to produce cell damage.

Floc formation due to cell wall breakage was observed with all three microalgae. *S. obliquus* and *C. sorokiniana* produced significant floc formations only at temperatures higher than 145°C, while *A. maxima* started to form aggregates at 105°C. Post-treatment floc formation due to exopolymers and intracellular compounds released during the treatment is an indication of cell wall breakage (González-Fernández et al., 2012). The low VSS/VS ratio of the homogenous mixture of *A. maxima* obtained at 165 °C indicate complete biomass solubilisation and maximum sCOD (100 mg gTS^{-1}). These results show that, in addition to the temperature of the treatment, the algal species and their characteristics are equally important when using thermal pre-treatments. Cellulose-free cells, such as cyanobacteria, achieve significantly higher cell damage at lower temperatures while ARB-enriched cells, such as green algae, require more than 165°C to achieve complete solubilisation.

3.1.2 Ultrasounds pre-treatment

When exposed to increasing amount of ultrasound energy during ultrasounds pre-treatment, all three algae released additional amounts of sCOD. The two green algae *S. obliquus* and *C. sorokiniana* showed a linear correlation between the energy input and the sCOD released (Figure 2). The highest sCOD increase occurred at 35 MJ kg TS^{-1} (U5): 346 mg gTS^{-1} for *S. obliquus* (10 fold) and 166 mg gTS^{-1} for *C. sorokiniana* (5 fold). However, both algae reported a limited solid solubilisation (VSS/VS), close to 20% (Table 3). In contrast, *A. maxima* reached 82% solubilisation at a lower energy input of 10 MJ $kgTS^{-1}$ (U3) showing small differences when subjected to higher energy treatments. Similar to what observed for the thermal treatments, *A. maxima* could be degraded more easily than the two green algae because of its lack of carbohydrates polymers/matrix and ARB components. Furthermore, *Arthrospira* sp. is characterised by the presence of septa and air vesicles in the cell wall structure making this alga particularly sensitive to the localised high pressures produced by ultrasonic treatment (Purcell et al., 2013).

In agreement with our results, when treating different mixtures of algae biomass, Alzate et al. (2012) reported different energy demands to achieve equal COD solubilisation for filamentous and single cells algae. *Microspora* sp., a filamentous algae, was efficiently treated using 50% less energy (~25 MJ kg^{-1}) than single cells algae *Acutodesmus obliquus*, *Oocystis* sp. and *Nitzschia* sp. Limited impact on the cell wall structure of single cell microalgae, despite the high energy used, was also reported by González-Fernández et al. (2012) who observed similar particle size distribution when treating *S. obliquus* at energy inputs ranging from 35.5 MJ kg^{-1} (equal to U5) to 129 MJ kg^{-1}. Our results suggest that ultrasound produced more structural damage on the cells of *A. maxima* whereas most of the sCOD released by the two green algae was the result of intracellular AOM escaping the partially damaged cell boundaries.

3.1.3 Enzymatic hydrolysis

During enzymatic pre-treatments, the action of low temperature thermal treatment (50°C for 24 hours) was combined with the catalytic activity of the enzymes. Enzymatic hydrolysis was performed using single enzymes and mixed enzymatic preparations. The enzymes were chosen taking into consideration the algae wall composition. The main components of *Scenedesmus* sp. and *Chlorella* sp. cell wall are sugars (24 – 74%), uronic acid (4 – 24%), proteins (2 – 11%) and glucosamine (0 – 15%), in addition to cellulose and hemicellulose (Blumreisinger et al., 1983). Whereas, the cell wall of *A. maxima*, a cellulose-free microalga, is mainly murein (peptidoglycan) layers covered by a coat of lipopolysaccharide (Tomaselli, 2007). Enzymatic mixtures released more than double soluble COD than single enzymes, (Figure 3). Cellulase plus pectinase mix (E1) and esterase plus protease mix (E2) were the most effective catalysts for all three algae followed by the single enzyme esterase (E5). The α-amylase (E3) was particularly active on *C. sorokiniana*, whereas pectinase (E4) mainly degraded *S. obliquus* suggesting a more selective action for these two enzymes being cell-algal and cell-wall component specific.

Between 150 and 250 U ml^{-1}, equal to 7.5 and 12.5 U gTS^{-1}, respectively, were used to maximise the sCOD released by all the enzymes tested (Figure 3). In particular, *S. obliquus* required 12.5 U $gTS^{-1}$of E2 to release up to 360 mg gTS^{-1} of sCOD, while comparable amounts of sCOD were released by *C. sorokiniana* (389 mg gTS^{-1}) and *A. maxima* (434 mg gTS^{-1}) at 7.5 U gTS^{-1}. Similar enzymatic activities (cellulase) were used by Yin et al. (2010) with *Chlorella* sp., suggesting that optimal enzymatic additions for microalgae with a low (10% w/w) and high (20% w/w) solid concentration close to 150 U ml^{-1} or 7.5 U gTS^{-1}. Although it was not possible to measure the VSS/VS ratio because of the enzyme's impact on the amount of total VS, the amount of sCOD released by the enzymes was similar to the amount released by the thermal treatment (TH165), suggesting a comparable solubilisation rate (35 – 45%).

Similarly to the other pre-treatments, the effect of the enzymes is linked to the algae cell wall composition. In agreement with previous works, cellulases performed well with all three algae (Yin et al., 2010; Fu et al., 2010; Harun et al., 2011; Zheng et al., 2011), while the mix of protease and esterase released higher amounts of sCOD than those previously reported (Sander and Murthy, 2009; Ehimen et al., 2013). Therefore, for an effective enzymatic hydrolysis it is necessary to take into account also the proteic and polysaccharidic components. The visual observation of the enzymatically pre-treated algae showed a significant change in colour from dark green to dark brown with no formation of algal floc, suggesting a different degradation mechanism from the other two thermal and ultrasound pre-treatments.

3.2 Treatment comparison: efficiency and cell wall breaking mechanisms

The AOM composition released by thermal hydrolysis at 165ºC (TH5) and ultrasonic pre-treatment at 35 MJ kg TS^{-1} (U5) was compared to the enzymatic one (average value between E1 and E2 at 7.5 U gTS^{-1}). The amount of soluble proteins (sP), carbohydrates (sC) and other compounds, including lipids (sL), released by the algal biomass changed significantly with each treatment and for each of the algal species investigated (Figure 4). The differences in composition of the soluble fraction indicate different mechanisms of action between the three processes tested. For single cell algae such as *S. obliquus* and *C. sorokiniana* most of the protein and lipid content of the biomass belongs to the intracellular AOM, while carbohydrates are the main constituent of the cell wall (Blumreisinger et al., 1983; Heaven et al., 2011). Releases of organics that are high in protein and lipid content suggest an efficient release of the AOM and cell wall breakage, whereas high sugar concentrations suggest efficient degradation and solubilisation of the cell wall constituents. Hence, the differences in composition can be used as a diagnostic indicator of the principle mechanism of action.

Thermal hydrolysis was the most effective pre-treatment in releasing proteins: for *S. obliquus*, the protein content released by thermal hydrolysis (80% of sCOD) was equal to 331 mg gTS^{-1} as BSA, 2.8 and 4.2 times higher than with ultrasound and enzymes, respectively. Similar results were obtained for *C. sorokiniana* (4.5 and 1.2 fold increase compared to ultrasounds and enzymatic hydrolysis respectively). No significant differences were observed with *A. maxima* across the three systems with an average value close to 50 mgBSA gTS^{-1} (~50% of sCOD after TH and U, 20% after enzymatic hydrolysis).

Ultrasounds released the highest amount of other/lipids, equal to 46% and 31% of the total sCOD for *S. obliquus* and *C. sorokiniana*, respectively.

In contrast, enzymatic hydrolysis was the most effective pre-treatment in releasing carbohydrates, almost certainly the product of cell wall degradation rather than breakages. In particular, with *S. obliquus*, enzymatic hydrolysis increased the soluble carbohydrate concentration to 135 mg gTS^{-1} from an average value of 3 mg gTS^{-1} (Table 2), 1.7 times more than TH, and 4.5 times more than ultrasound. Similarly, with *C. sorokiniana*, the soluble carbohydrates concentration increased to 42 mg gTS^{-1} as glucose with ultrasound, and to 65 and 95 mg gTS^{-1} after TH and enzymes, respectively. *A. maxima* showed similar low concentrations after each treatment (5% of sCOD as an average value).

A. maxima has a different distribution of proteins, carbohydrates and lipids between the internal AOM and the membrane wall (Heaven et al., 2011; Tomaselli, 2007). A high lipid content suggests membrane degradation and solubilisation, whereas high protein and carbohydrate contents indicate the release of intracellular AOM. In all cases, enzymes seem to act preferentially on cell wall components, while thermal and ultrasound treatments produce AOM release following cell wall degradation.

The difference in action mechanisms was confirmed by microscopic analysis of untreated and treated green algae cells (Figure 5). The TEM images of untreated cells clearly showed all

main microalgae cell components, such as the cell wall, the nucleus and thylakoids filling most of the cytoplasm (Figure 5, SO1 and CS1). Thermal hydrolysis expanded and partially disaggregated the cell wall structure causing the release of internal AOM in the media. This is clearly shown in Figures 5 SO2 and CS2 by the loss of cell turgidity and the appearance of empty/clear areas inside the cells boundaries. As previously reported by Choi et al. (2011), ultrasound treatment caused a loss of external cell boundaries (small black points surrounding the cell wall) and release of AOM into the media (Figures 5, SO3 and CS3). Loss of external cell boundaries and cell turgidity were less evident after enzymatic hydrolysis, despite the high amount of AOM released (clear spaces inside the cells boundaries and high measured sCOD). The less distorted cell structure suggests a more specific degradation and solubilisation of the cell wall components (e.g. production of mono-, di-saccharides as products of an efficient enzymatic activity) (Figure 5, SO4 and CS4). Similar observations were reported by Rodrigues and da Silva Bon (2011). Investigating the sugar composition of the material released by enzymatic hydrolysis of *Chlorella* sp., the authors were able to ascertain the cell wall composition of different algae strains demonstrating specific enzymatic activities on cell wall. No clear TEM images were obtained for *A. maxima* since the harsh conditions used for the TEM sample preparation degraded the cell structure.

3.3 Pre-treatments effect on energy recovery

3.3.1 Anaerobic digestion batch test

Anaerobic digestion of untreated *S. obliquus*, *C. sorokiniana* and *A. maxima* produced up to 88 ± 2, 118 ± 5 and 60 ± 6 ml g VS^{-1} of methane, respectively. While the yields of green algae compared well with data available in literature, *A. maxima* yields were low. This is probably caused by inhibition on the microbial population of residual salts from the algae growth medium (Chen et al., 2008).

All pre-treated biomass showed improvements in methane yields with the only exception of *A. maxima* when treated at 105°C and 120°C (Table 3). The highest methane production was obtained with enzymatic hydrolysis. When using a mixture of enzymes the methane yields increased to 1050 ± 201, 775 ± 253 and 1197 ± 254 ml gVS^{-1} for *S. obliquus*, *C. sorokiniana* and *A. maxima*, respectively. With thermal treatments, the maximum methane improvement occurred at 165°C with *S. obliquus* yielding 268 ± 2 ml g VS^{-1} (+ 208%) followed by *A. maxima* (+ 70%) and *C. sorokiniana* (+ 98%). Compared to the thermal treatments, the ultrasound produced 96% methane improvement with *S. obliquus*, 38% with *A. maxima* and 42% with *C. sorokiniana*. Using similar digestion conditions, Alzate et al. (2012) reported 55% methane production improvement, from 198 ± 9 to 307 ± 9 ml gVS^{-1}, digesting a mixture of natural algae pre-treated at 170°C. When treated with ultrasound (10 - 57 MJ kg^{-1}), the same algal mixture showed methane yield increments between 6 and 13%, confirming that thermal treatments can be more effective than ultrasounds in enhancing microalgae digestibility.

With enzymatic hydrolysis, cellulase plus endogalacturonase (E1) and esterase plus protease (E2), improved the methane production up to 12 times, with yields ranging between 477 and 730 ml gVS^{-1}. Using a 1:1 (E1:E2) enzymatic mixture, further increases were achieved: 16-fold increase in methane production with *A. maxima*, 6.7-fold with *S. obliquus* and 3.5-fold with *C. sorokiniana*.

In agreement with other reports on AD feedstock pre-treatments, digestion improvements were proportional to the COD solubilisation achieved (Cho et al., 2013a). However, similar amounts of sCOD and VS released by the same algae produced different methane yields when pre-treated with the different processes (Figure 6). For instance, with *C. sorokiniana*, sCOD concentration of 200 mg gVS^{-1} produced between 150 and 200 ml gVS^{-1} of methane after thermal or ultrasound pre-treatment (Figure 6 A) and 400 ml gVS^{-1} after enzymatic hydrolysis

(Figure 6 B). Similar results were observed with *S. obliquus* and *A. maxima* at 450 mg sCOD and 1300 mg sCOD, respectively (Figure 6).

These results echo those reported in Section 3.2 explaining the action mechanism of the pre-treatments. The higher yields obtained after enzymatic hydrolysis were the direct consequence of efficient cell wall biochemical degradation which enabled (1) removal of the compounds able to resist bacterial degradation and limiting the methane yields (cellulose and ARB) and, depending on the algae species, (2) release of a higher amount of energetically valuable components such as sugars and lipids. In contrast, the high intracellular AOM released after thermal and ultrasonic treatments was the result of cell wall breakage only which did not allow the solubilisation of bacterial resistant or inhibitory compounds.

3.3.2 Pre-treatments energy balance

In most of the tested conditions, the additional methane production was not sufficient to balance the energy required to pre-treat the biomass. Data plotted on a log-log scale chart (Ei/Eo ratio vs biogas increment) shows three clusters for the different pre-treatments (Figure 7). The enzymatic pre-treatment results are located at the bottom right part of the graph representing the most energetically balanced conditions, as the biogas production over balanced the energy input (+15 kJ $kgVS^{-1}$). The central part of the diagram is predominantly populated by thermally treated biomass and distributed across the neutral energy balance line (log Ei/Eo = 1). Higher energy inputs (higher temperatures), were responsible for higher methane yields and a more positive energy balance. Results related to the ultrasound are located on the top left part of the diagram, with an average net energy balance of close to -30 MJ kg VS^{-1}. In this case, low specific energy inputs were more energetically efficient even with methane increments lower than 50%. To compare, the results reported by Cho et al. (2013a) on *Scenedesmus* sp. and *Chlorella* sp. biomass pre-treated with ultrasound (39 - 234 MJ kg VS^{-1}) and thermal treatment fit the clusters of Figure 7, confirming the different energy

impacts of the two pre-treatments. The highest negative value of energy was that of thermally pre-treated *A. maxima*. This was mainly due to the release of lower energy content compounds compared to those released after ultrasounds and enzymatic hydrolysis.

4. Conclusions

Enzymatic hydrolysis enabled the solubilisation of the cell wall constituents. This allowed a higher biogas production compared to thermal and ultrasound pre-treatments responsible for physical cell wall degradation (deformation and breakage).

ARB-enriched algae (e.g. *Chlorella* sp. and *Scenedesmus* sp.) required more energy intensive pre-treatments (higher temperatures, higher specific energy and higher enzymatic dosages) than cellulose free microorganisms (e.g. *Arthrospira* sp.).

Although the current study is based on small batch experiments, the findings suggest the key role of pre-treatments in the optimisation of biogas production from microalgae. Of the methods currently used in industry to pre-treat organic waste and sludge, ultrasounds produced the most energetically imbalanced process while high temperature thermal hydrolysis and enzymatic hydrolysis were the most energetically efficient. Further investigation into the effects of enzymatic additions on AD is strongly recommended to validate their positive impact on the process and their costs/feasibility for large scale applications.

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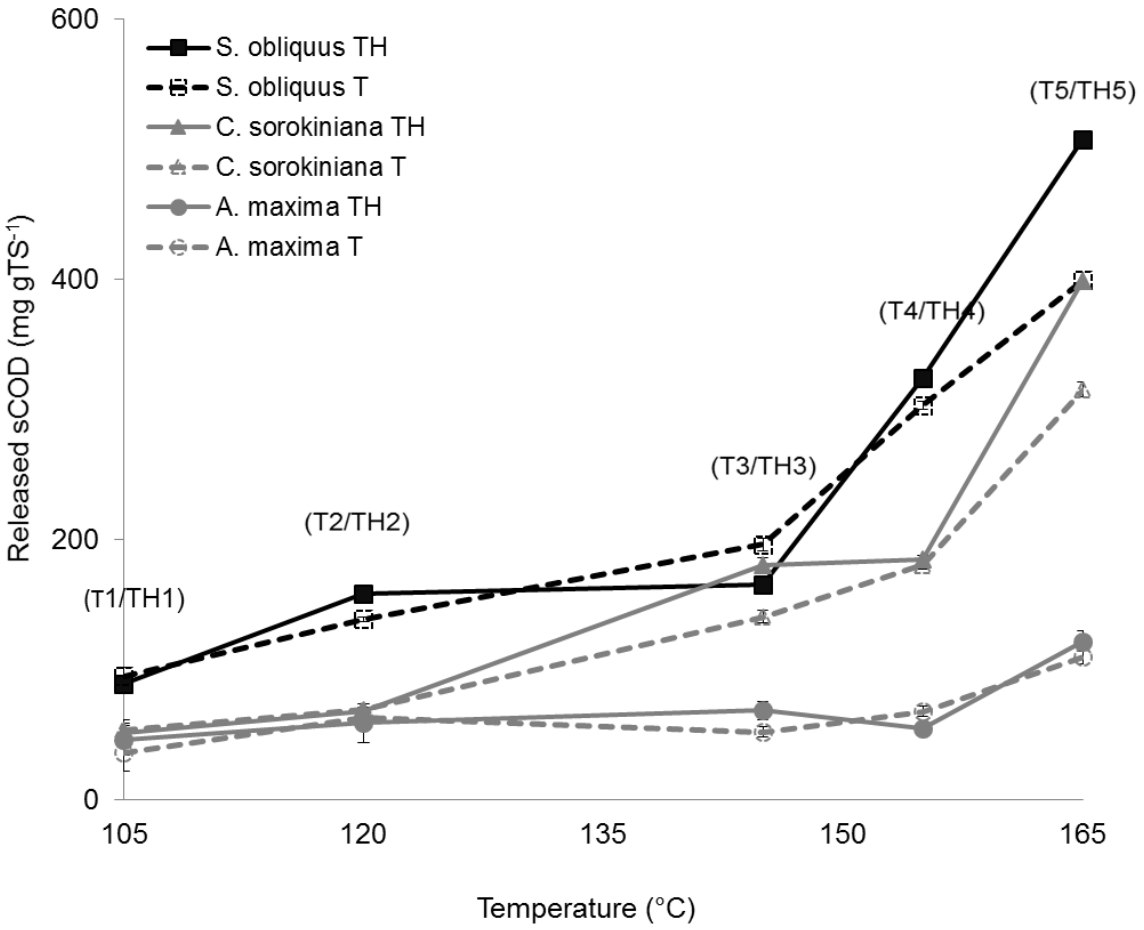

Figure 1 Thermal (T) and Thermal Hydrolysis (TH) pre-treatments. Soluble COD released by *S. obliquus*, *C. sorokiniana* and *A. maxima* treated at increasing temperatures and pressure ($p<0.05$).

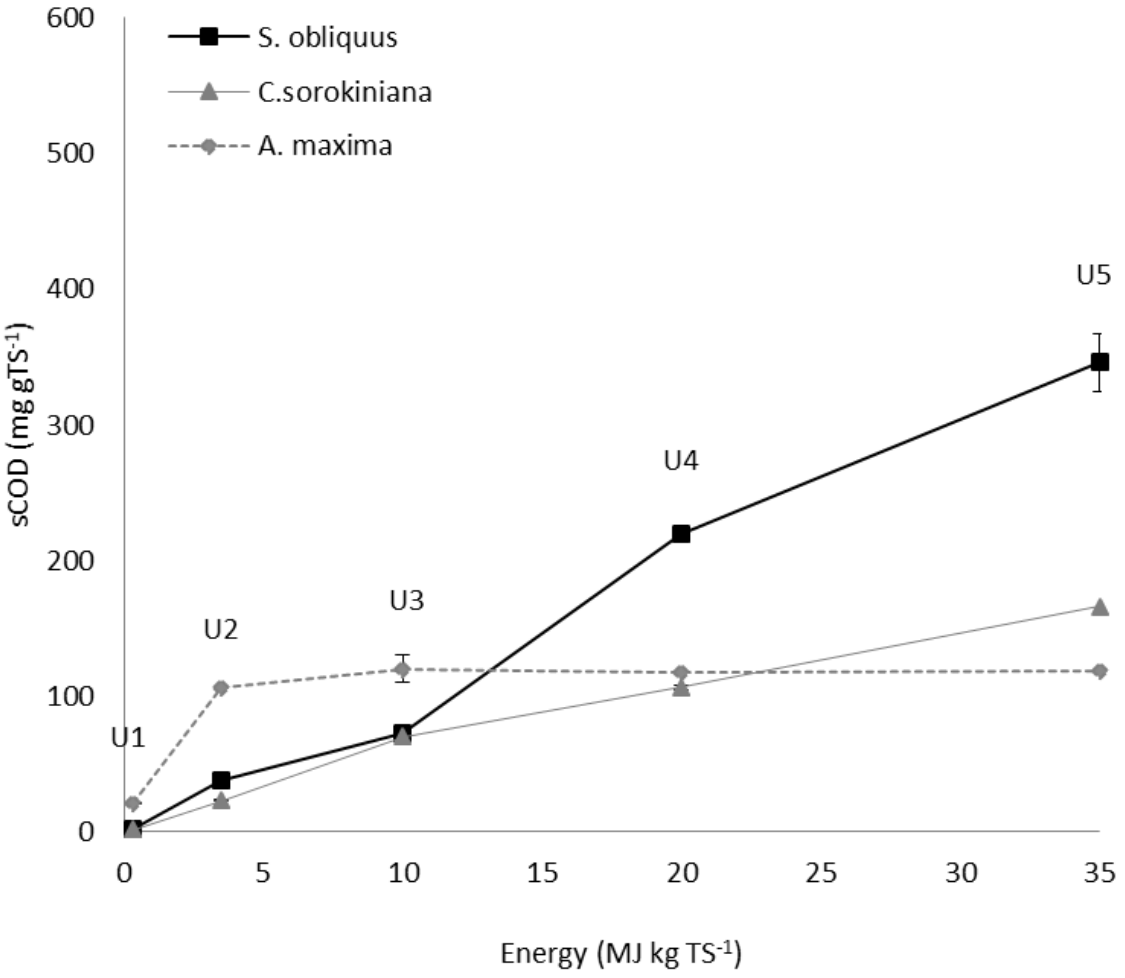

Figure 2 Ultrasound pre-treatment. Soluble COD released by *S. obliquus*, *C. sorokiniana* and *A. maxima*, exposed to increasing amount of energy ($p<0.05$).

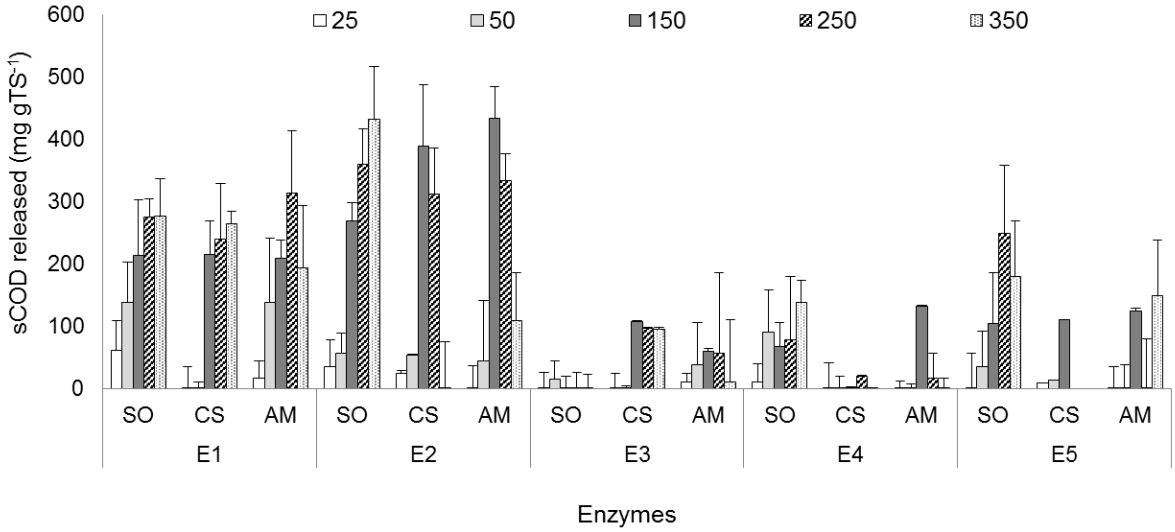

Figure 3 Enzymatic hydrolysis pre-treatment. Soluble COD released by *S. obliquus* (SO), *C. sorokiniana* (CS) and *A. maxima* (AM*)* treated with different enzymes (E1-E5) at increasing activity (25, 50, 150, 250, 350 U ml^{-1}) ($p<0.05$). All the samples have been subtracted of the sCOD released by the seed and by the enzymes.

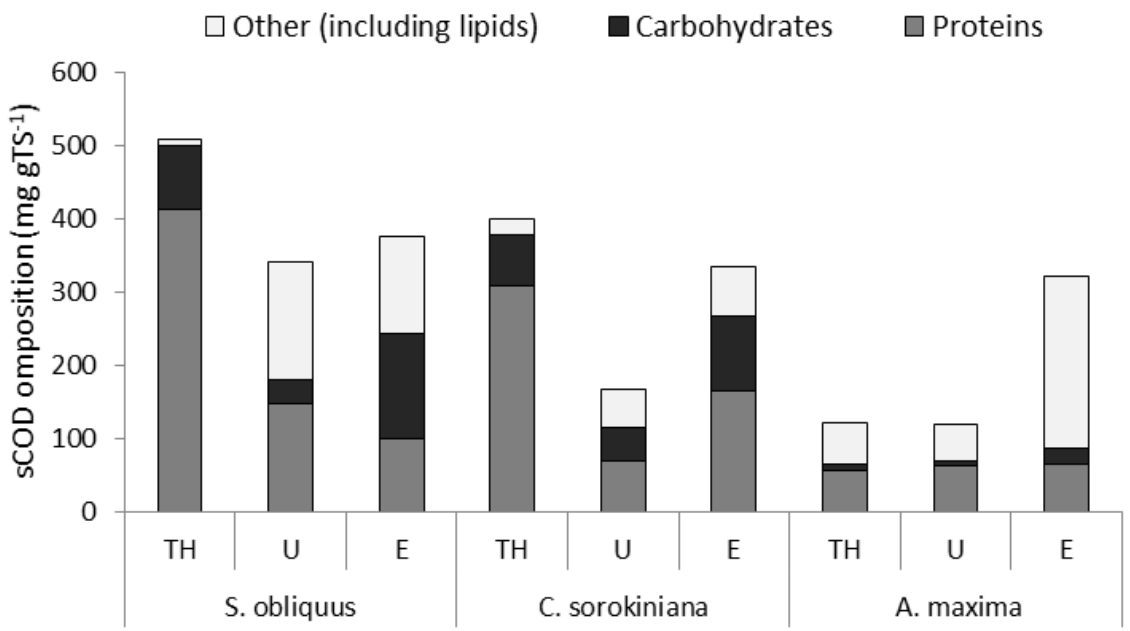

Figure 4 Comparison of the soluble COD content released by *S. obliquus*, *C. sorokiniana* and *A. maxima* after thermal hydrolysis (TH), ultrasound (U) and enzymatic (E) pre-treatment.

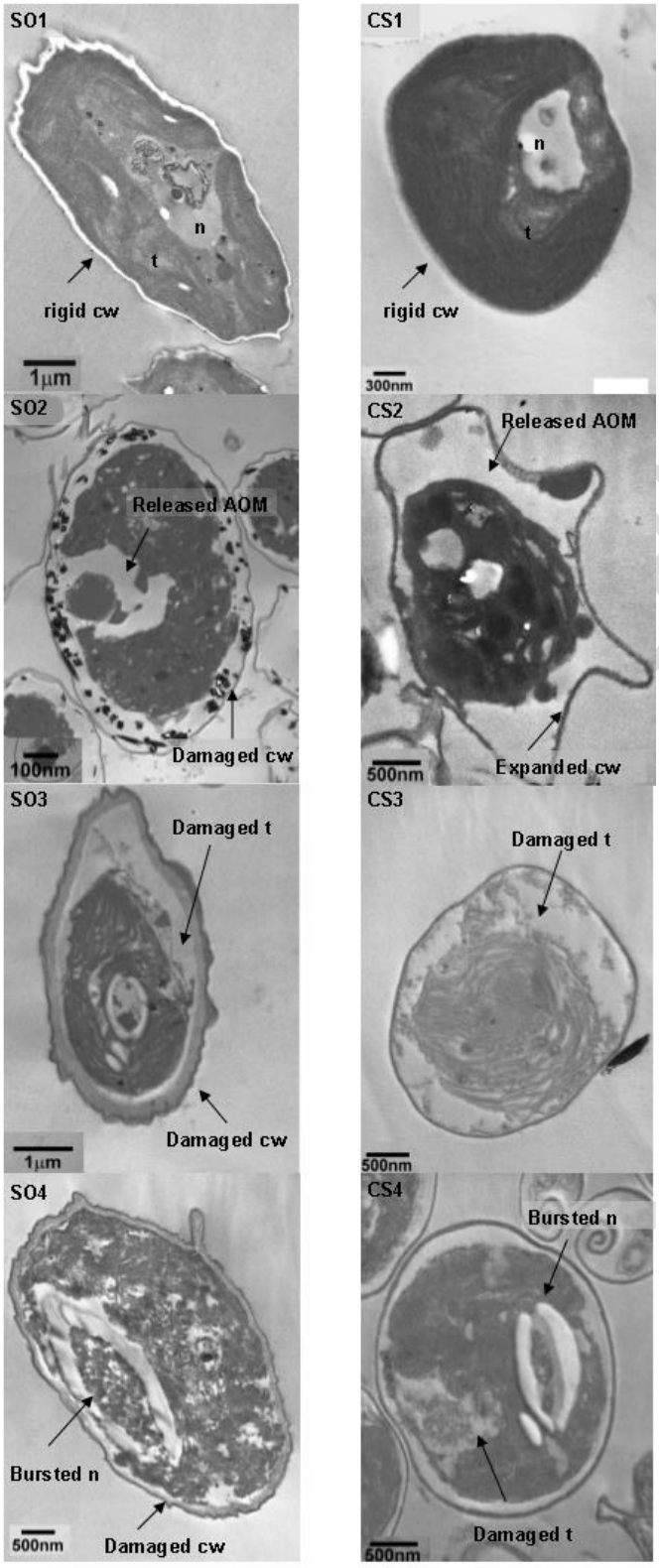

Figure 5 TEM pictures of *S. obliquus* (SO) and *C. sorokiniana* (CS) cells, untreated (1) and after thermal hydrolysis (2), ultrasound (3) and enzymatic (4) pre-treatment. Nomenclatures: cw= cell wall; n= nucleus, t= thylakoids.

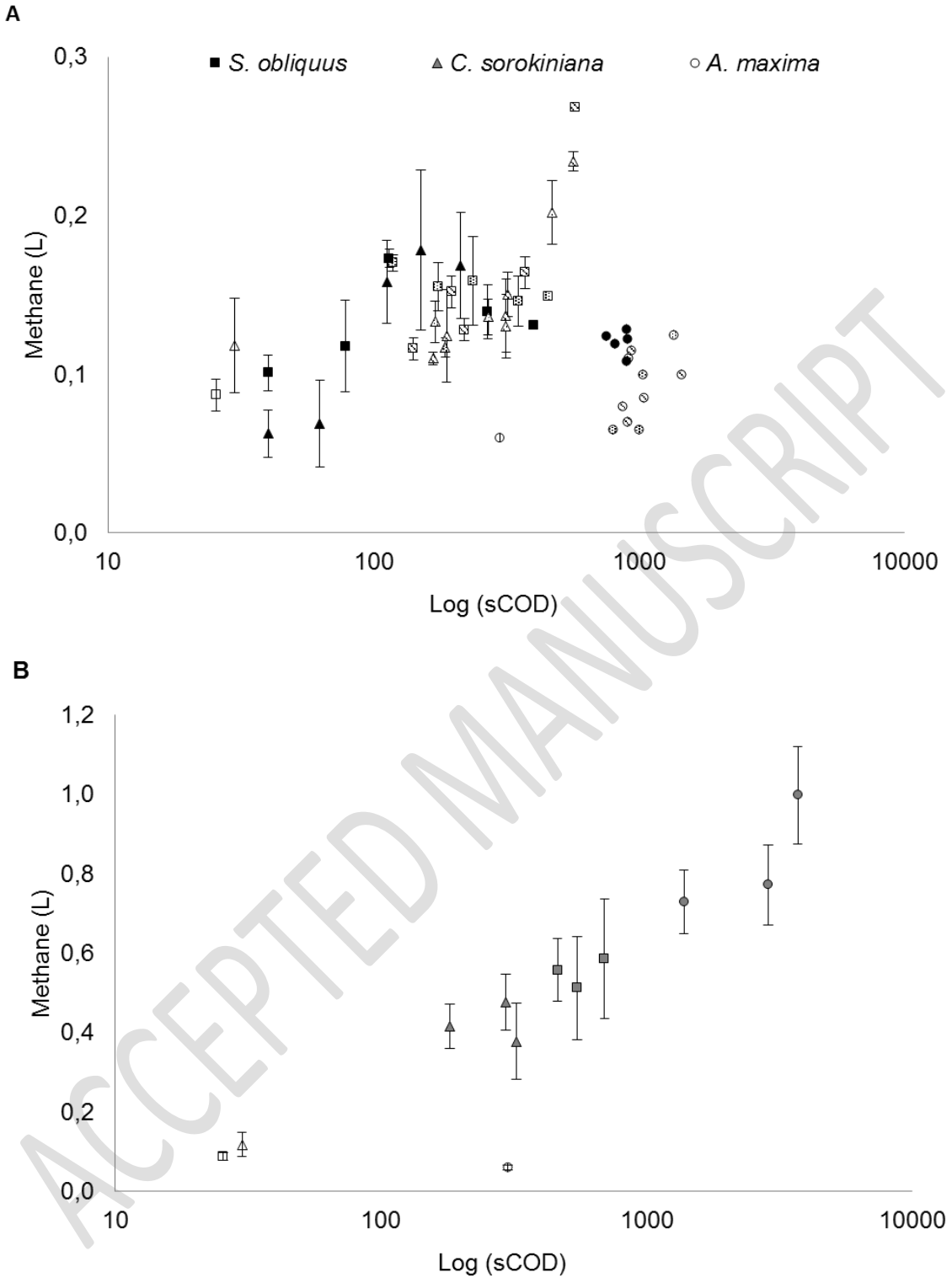

Figure 6 Methane productions per available sCOD (normalised on gVS) after (A) physical pre-treatments including thermal (dotted markers), thermal hydrolysis (lined markers) and ultrasound (full markers), and (B) enzymatic hydrolysis. Empty markers represent untreated biomass.

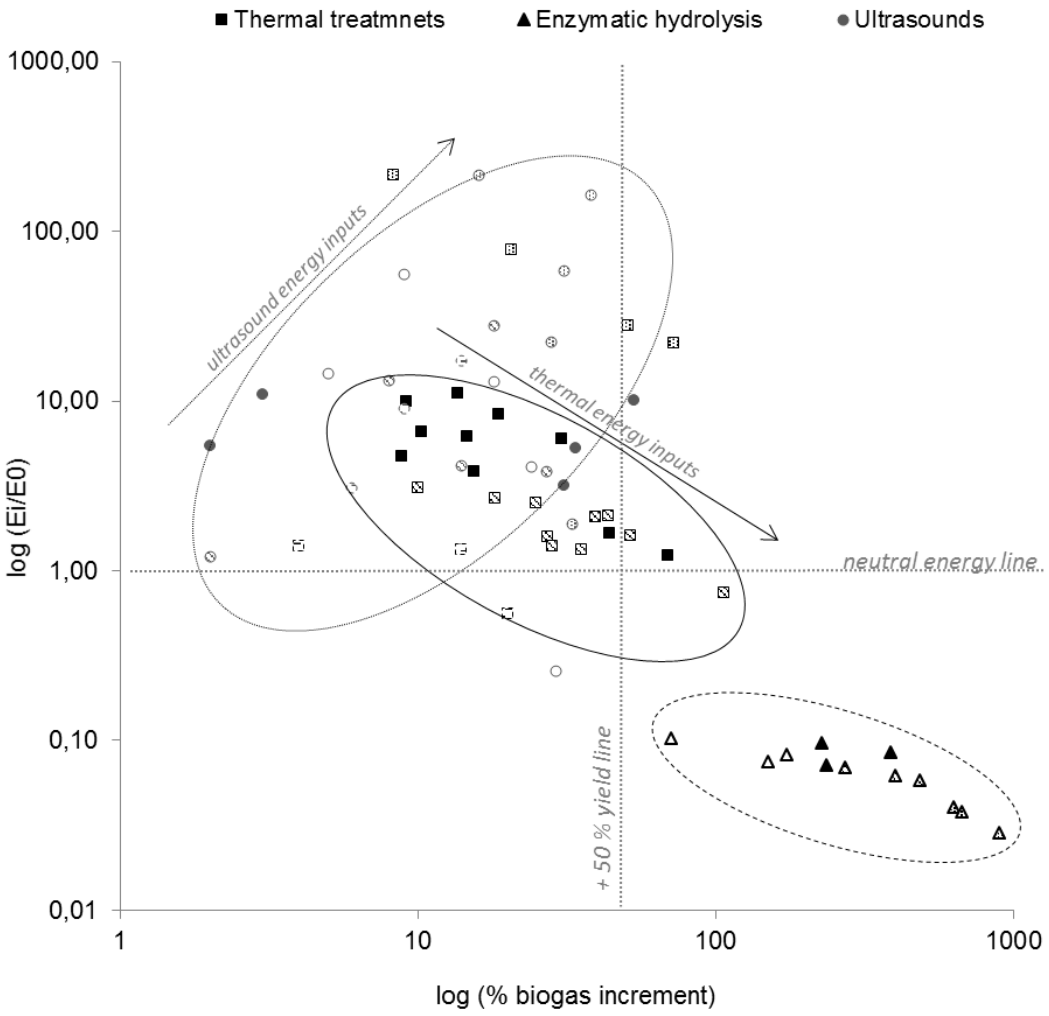

Figure 7 Energy balance of all the treated biomass and the related increments in biogas: *S. obliquus* (lined markers), *C. sorokiniana* (full markers) and *A. maxima* (dotted markers); discontinue markers line (Cho *et al.*, 2013)

Table 1 List of enzymes and their characteristics.

Enzymes	Commercial name	Composition	Supplier
E1	Depol^{TM} 40L	Cellulase 1,200 U g^{-1} + Endogalactouronase 800 U g^{-1}	Biocatalysts Ltd, UK
E2	Lipomod^{TM} 957	Esterase 3,600 U g^{-1} + Protease 90 U g^{-1}	Biocatalysts Ltd, UK
E3	Depol^{TM} 220L	Alpha amylase 25,000 U g^{-1}	Biocatalysts Ltd, UK
E4	Pectinase P2611	Pectinase 3,800 U g^{-1}	Sigma Co Ltd, UK
E5	Lipomod^{TM} 166P	Esterase 5,220 U g^{-1}	Biocatalysts Ltd, UK

Table 2 Microorganisms characterisation.

Microorganisms	description			soluble COD[a] (mg gTS^{-1})			
	taxionomy	shape	size	total	as proteins	as carbohydrates	as other including lipids
Scenedesmus obliquus	green algae	spindle	6 w; 10.5 l	25 ± 3	3.7 ± 0.5	1.6 ± 0.3	17 ± 1
Chlorella sorokiniana	green algae	spherical	4.5 Ø	95 ± 2	2.4 ± 0.5	1.8 ± 0.5	90 ± 1
Arthrospira maxima	cyanobacteria	filament	4.5 Ø; 300 l	300 ± 10	103 ± 8	74 ± 3	123 ± 5

[a]average value from different batch culture (mean±SD).

Table 3 Summary table: biogas production pre and post treatment (mean±SD) of all tested biomass. Bold numbers represent a positive energy balance.

Treatment	*Scenedesmus obliquus*					*Chlorella sorokiniana*					*Arthrospira maxima*				
	VSS/VS	Biogas (ml/gVS$_{add}$)	Increase[a] (%)	CH_4[b] (%)	E_i/E_0	VSS/VS	Biogas (ml/gVS$_{add}$)	Increase[a] (%)	CH_4[b] (%)	E_i/E_0	VSS/VS	Biogas (ml/gVS$_{add}$)	Increase[a] (%)	CH_4[c] (%)	E_i/E_0
Control[d]	0,95	265±10	-	60	-	0,96	273±37	-	62	-	1,16	185±20	-	-	-
T1	0,83	270±11	10	60	3,09	0,87	297±27	9	55	4,73	0,78	129±20	-11	-	-
T2	0,87	359±14	35	60	1,33	0,96	310±68	14	66	11,12	0,79	127±24	-12	-	-
T3	0,78	337±39	27	65	1,58	0,61	356±16	30	58	5,96	0,78	219±06	51	-	27,64
T4	0,83	370±35	40	63	2,06	0,54	313±54	15	60	6,14	0,78	175±14	21	-	78,06
T5	0,6	381±14	44	68	2,11	0,63	393±50	44	69	1,67	0,37	250±5	72	-	21,80
TH1	0,85	331±85	25	60	2,51	0,79	301±11	10	57	6,54	0,77	157±09	8	-	212,97
TH2	0,82	340±14	28	67	1,39	0,86	298±20	9	58	10,01	0,71	138±12	-5	-	-
TH3	0,75	313±23	18	63	2,69	0,72	324±53	19	60	8,34	0,78	173±18	19	-	85,42
TH4	0,89	404±6	52	60	1,60	0,53	315±41	15	64	3,86	0,77	235±9	62	-	24,16
TH5	0,58	548±2	107	70	**0,75**	0,57	461±4	69	69	1,22	0,35	200±40	38	-	43,60
U1	0,99	266±27	2	64	1,21	0,95	249±12	2	62	5,43	0,75	206±3	33	-	1,88
U2	0,99	299±24	14	60	4,18	0,88	252±40	3	65	10,86	0,49	199±21	28	-	22,22
U3	0,89	333±16	27	73	3,83	0,89	320±25	31	66	3,17	0,18	203±22	31	-	58,19
U4	0,90	292±12	8	63	13,21	0,90	368±12	34	64	5,26	0,15	180±5	16	-	214,87
U5	0,89	307±15	18	65	27,85	0,83	375±35	53	65	10,06	0,13	214±18	38	-	162,94
E1[e]	-	1425±224	403	63	**0.06**[h]	-	1158±116	236	46	**0.07**[h]	-	1461±173	630	-	**0.04**[h]
E2[f]	-	1065±201	273	58	**0.07**[h]	-	868±253	227	56	**0.10**[h]	-	1545±201	672	-	**0.04**[h]
E1:E2[g]	-	1669±63	485	63	**0.06**[h]	-	1292±148	387	60	**0.09**[h]	-	1996±254	898	-	**0.03**[h]

[a]compared to the control batch experiment performed with every new batch of algae; [b]methane content at the end of the batch digestion; [c]not directly measured over time and assumed equal to 60%; [d]average value between all control experiment performed; [e]inoculum+enzymes biogas production equal to 102 ± 19 ml gVS^{-1}; [f]inoculum+enzymes biogas production equal to 121 ± 23 ml gVS^{-1}; [g]inoculum+enzymes biogas production equal to 124 ± 22 ml gVS^{-1}; [h]energy to produce the enzymes was exclude from the calculation;