Renewable energy- evaluation of low energy demand pre-treatments to optimize methane production from microalgae

Yanghanzi Zhang^{1*}, Gary S. Caldwell², Paul J. Sallis¹

¹ School of Engineering, Newcastle University, Cassie Building, Claremont Road, Newcastle upon Tyne, NE1 7RU, UK

² School of Natural and Environmental Sciences, Newcastle University, Ridley Building, Claremont Road, Newcastle upon Tyne, NE1 7RU, UK

*y.zhang33@newcastle.ac.uk

Abstract: Anaerobic digestion (AD) of microalgae is a sustainable process to produce methane-rich biogas for bioenergy production. However, the rigid cell walls of microalgae protect them against the attack from hydrolytic bacteria, and consequently prevent efficient biodegradability and lower methane production. In this study, the effects of low energy demand enzymatic and low-temperature thermo-alkaline pre-treatments on microalgae AD were investigated in batch biochemical methane potential (BMP) tests. The results found that methane yields were significantly enhanced by both pre-treatments. Microalgae *C. vulgaris* after enzymatic pre-treatment enhanced methane yields the most, by 22–162%, whilst *C. vulgaris* pre-treated by thermo-alkaline pre-treatment improved methane yields by 4–26%. In enzymatic pre-treatment, *C. vulgaris* pre-treated with mixed enzymes showed higher methane yields compared to single enzymes. In low-temperature thermo-alkaline pre-treatment, the level of methane yield enhancement depended on alkaline dosage and pre-treatment temperature, but the high alkaline dosages were associated with limitations such as a prolonged lag phase in the digestion process. From an energy viewpoint, both pre-treatments showed positive energy balances for the majority of experimental conditions, and therefore both pre-treatments are considered to be energetically efficient methods to pre-treat microalgae for methane production via AD.

1. Introduction

The depletion of fossil fuels in the energy sector has led to increase levels of greenhouse-gas (GHG) emissions that represent nearly 66% of total anthropogenic GHG emissions [1]. GHG warm the atmosphere, seriously affect rainfall patterns, cause the retreat of glaciers and sea ice, and raise sea levels due to climate change [2]. Therefore, in order to avoid further escalation of climate change, effective action should be taken in the energy sector, particularly using clean and new energy sources instead of conventional fossil fuels. Biomass energy has been recognised as one of options that will play an important role in future energy scenarios, and a recently emerging strategy is to convert biomass into clean energy fuels via biorefinery technology [3]. Currently, biorefineries are mainly based on energy crops, however, the main concern in using energy crops for the bioenergy market is that their production on agricultural land may detrimentally affect global food supplies [4]. Compared to energy crops, using microalgae for biofuel production has become an alternative in order to reduce competition with food industry. Moreover, microalgae may be grown independently of arable land, and their productivity may potentially reach 100–150 tonnes/ha/year, which is 10–15 times higher than that of conventional energy crops [5].

Microalgae are able to generate different types of biofuels such as biodiesel, biogas, bioethanol, biohydrogen and bio-oil [6-8]. Among the latter, biogas and biodiesel are the most common types of biofuels because they can be used to replace natural gas and petroleum diesel, respectively [9]. Indeed, most contemporary studies focus using microalgae to produce biodiesel. However, when compared to the biodiesel production process, anaerobic digestion (AD) is a more straightforward biological process used to produce bioenergy

without biomass concentration and drying, lipid extraction and fuel conversion. Moreover, most microalgae macromolecules (carbohydrates, proteins and lipids) can be fermented via AD to generate methane-rich biogas. Therefore, the microalgae AD process for bioenergy production may be less complex.

During the AD process, the feedstock's cell walls are firstly degraded by the extracellular enzymes of hydrolytic bacteria, but the duration and rates of the hydrolysis process strongly depends on the feedstock type [10]. Some microalgae strains are able to thrive in wastewater effluents or severe environments such as high salinity and have chemically and structurally robust cell walls [11]. Generally, microalgal cell walls have outer and inner layers. The outer layer of the microalgal cell wall is composed of specific matrix polysaccharides such as pectin, chitin agar or algaenan, while the inner layer contains microfibrillar cellulose as well as hemicellulose and glycoproteins [12]. Similar to plants, the proportions of different compositions in microalgal cell wall are 25–30% of cellulose, 15–25% of hemicellulose, 35% pectin and around 5–10% glycoproteins [13]. Cellulose is a complex, water-insoluble polysaccharide with a rigid linear structure [14], and this structurally stable cellulose together with hemicellulose, proteins and other compounds forms a rigid cell wall that protects the microalgae against attack from hydrolytic bacteria, and consequently prevents efficient biodegradability and lowers methane yields [15]. Therefore, the hydrolysis was the rate-limiting step for microalgae, and effective pre-treatment are required to disrupt the microalgal cell wall in order to enhance methane production.

Previous investigations have tested a wide range of pre-treatments involving high energy demand, including mechanical (such as ultrasound), thermal and high-temperature thermo-chemical methods to enhance methane

production from microalgae [16-17]. However, these pre-treatments are associated with high energy inputs, which can be higher than the amount of energy recovered in the form of additional methane. These pre-treatments are energetically unbalanced, although they have been reported to improve methane yields efficiently. Therefore, research interest has been directed towards the use of low-energy demand pre-treatments for microalgal biomass. Enzymatic pre-treatment requires a mild pre-treatment temperature, and therefore it is an energetically competitive method. Moreover, the use of enzymes can reduce the possibility of releasing by-products that might inhibit the subsequent AD process [15]. However, previous studies mostly focus on the pre-treatment of microalgae using single enzymes, and only few investigation have considered the effect of mixed enzymes on microalgal AD, especially cellulase mixed with α -amylase.

Another energetically competitive method is alkaline pre-treatment, and this also requires low energy inputs compared to thermal and ultrasonic pre-treatments [18]. However, in order to improve the pre-treatment efficiency, alkaline pre-treatments are usually combined with high temperatures (>100 °C) in pre-treating microalgal biomass [18-19]. Moreover, previously, NaOH has been studied the most for the pre-treatment of microalgal biomass for methane production [13, 17, 18]. However, as the high cost pre-treatment reagent, the drawbacks in using NaOH should be considered, such as it may increase the concentrations of Na^+ in the digestate, which could be potentially be toxic to the methanogens [20]. Calcium hydroxide ($\text{Ca}(\text{OH})_2$) is less expensive than other types of alkaline and can be regenerated using established lime kiln technology [21]. Previously, lime has been used to pre-treat other substrates such as solid wastes and smooth cordgrass to improve their biodegradability and methane production via the AD process [22-23]. However, few investigations have used lime to pre-treat microalgae. Therefore, from the energy viewpoint, there is a need to investigate the effect of low-temperature thermo-alkaline pre-treatment of microalgal biomass for AD, especially using $\text{Ca}(\text{OH})_2$ as the pre-treatment reagent.

This work aimed to investigate the effect of low energy demand enzymatic and low-temperature thermo-alkaline pre-treatments on the degree of solubilisation of the microalgal biomass, and the subsequent methane yields in batch BMP tests. For enzymatic pre-treatment, the effects of different dosages and types of enzymes were investigated. For low-temperature thermo-alkaline pre-treatment, the effects of $\text{Ca}(\text{OH})_2$ dosages, pre-treatment temperature and pre-treatment time were investigated

2. Materials and Methods

2.1. Microalgae and inoculum

The *Chlorella vulgaris* strain (CCAP 211/63) was obtained from Culture Collection of Algae and Protozoa, UK. The *C. vulgaris* strain was cultured in Bold's Basal Medium in 10 L Nalgene carboys at 19 °C under artificial light with a 16:8 light dark photoperiod [24]. After 30 days of culture, microalgae were first concentrated by sedimentation for 48 hours at 4 °C, and then the supernatant was discarded. The remaining microalgae were centrifuged at $3392 \times g$ for 10 minutes in 8×45 mL sterilized centrifuge tubes (VWR, UK).

The supernatant was discarded and the pellet was re-suspended with 2 L of distilled water to remove the culture medium. The concentrated microalgae was characterised by its total solids (TS), volatile solids (VS), total chemical oxygen demand (COD_t) and soluble chemical oxygen demand (COD_s), carbohydrate and protein content (Table 1). In order to prevent prior degradation and significant changes in soluble carbon, the concentrated microalgae were stored at 4 °C for up to 2 weeks prior to AD tests.

Table 1 Characterisation of microalgae *Chlorella vulgaris*

Parameters	Value
TS (g/L)	2.0 ± 0.2^a
VS (g/L)	1.9 ± 0.2
VS/TS (%)	91.9 ± 1.9
COD_t (g/L)	2.7 ± 0.9
$\text{COD}_s/\text{COD}_t$ (%)	2.7 ± 2.1
Total Protein (% VS)	39.2 ± 4.8
Total Carbohydrate (% VS)	27.6 ± 3.2

^a Mean $\pm$ SD, n=4.

The anaerobic seed inoculum was collected from a manure-based farm anaerobic digester located at Cockle Park Farm, Northumberland, UK. The TS and VS of the anaerobic inoculum were 12.2 ± 0.1 and 6.6 ± 0.1 g/L, respectively.

2.2. Experimental design

Enzymatic pre-treatment was designed to evaluate the effect of concentrations and types of enzyme on hydrolysis efficiency in terms of solubilisations of COD, carbohydrate and protein; and methane yields. There were five types of enzyme tested in this study including cellulase only (C), protease only (P), amylase only (A), mixtures of cellulase plus protease (CP) and cellulase plus amylase (CA). For mixed enzymes, the mixture ratio of the two enzymes was set at 1:1. The tested enzyme concentrations were 0.5 and 1.0 % v/w. These concentrations were selected based on previous studies [25-26]. In order to catalyze the activity of the enzymes, pre-treatment conditions were set at 55 °C for 24 hours [25, 27]. After 24 hours pre-treatment, the samples were heated in an oven at 75 °C for 15 minutes to deactivate the enzymes [15]. A summary of the experimental design for enzymatic pre-treatment is shown in Table 2.

Table 2 Summary and coding of the experimental design for enzymatic pre-treatment trials.

Enzyme concentration (% v/w)	Experimental groups*				
0.5	C1	P1	A1	CP1	CA1
1.0	C2	P2	A2	CP2	CA2

*Treatment coding: 1-2 corresponds to the concentrations of enzymes, C=cellulase, P= protease, A= α -amylase, CP = cellulase mixed with protease, CA= cellulase mixed with α -amylase

The low-temperature thermo-alkaline pre-treatment was designed to use lime ($\text{Ca}(\text{OH})_2$) to pre-treat *C. vulgaris* at two low temperatures of 37 and 55 °C at different dosages (0, 10 and 15% w/w). These dosages were selected based on previous studies [6, 13]. The effect of pre-treatment time at 24 or 48 hours on hydrolysis efficiency and methane yields was also evaluated. The experimental design is shown in Table 3.

Table 3 Summary and coding of the experimental design for low-temperature thermo-alkaline pre-treatment trials.

$\text{Ca}(\text{OH})_2$ dosage (% w/w)					
0		10		15	
L1A*	L1B	L2A	L2B	L3A	L3B
H1A	H1B	H2A	H2B	H3A	H3B

*Treatment coding: 1-3 corresponds to the dosage of $\text{Ca}(\text{OH})_2$, L= low pre-treatment temperature of 37 °C, and H=high pre-treatment temperature of 55 °C; A=24 hours of pre-treatment time, B=48 hours of pre-treatment time

2.3. Evaluation of hydrolysis efficiency

The solubilisation of COD is a measure widely used to determine the efficiency of pre-treatments [15, 28], and the solubilisation of COD (% COD) was calculated according to Equation 1.

$$\% \text{ COD} = \frac{\text{COD}_{sp} - \text{COD}_{so}}{\text{COD}_t - \text{COD}_{so}} \times 100\% \quad (1)$$

where COD_{sp} is the concentration of the soluble COD of pre-treated microalgae, COD_{so} is the concentration of the soluble COD of untreated microalgae, and COD_t is the total concentration of COD of microalgae.

C. vulgaris is characterised by a carbohydrate-based cell wall, which is mainly composed of cellulose (25–30%) and hemicellulose (15–25%) [29–30]. Moreover, since microalgae are rich in protein contents, to further evaluate the efficiency of pre-treatments, solubilisations of carbohydrate (% CH) and proteins (% PT) were also calculated based on Equations 2 and 3.

$$\% \text{ CH} = \frac{\text{CH}_{sp} - \text{CH}_{so}}{\text{CH}_t - \text{CH}_{so}} \times 100\% \quad (2)$$

$$\% \text{ PT} = \frac{\text{PT}_{sp} - \text{PT}_{so}}{\text{PT}_t - \text{PT}_{so}} \times 100\% \quad (3)$$

where CH_{sp} and PT_{sp} are the concentrations of soluble carbohydrate and protein of pre-treated microalgae, CH_{so} and PT_{so} are the concentrations of soluble carbohydrate and protein of untreated microalgae, and CH_t and PT_t are the total concentrations of carbohydrate and protein of microalgae.

2.4. Biochemical methane potential (BMP) test

Batch BMP tests were performed based on the guidelines recommended by [33], using glass bottles with a capacity of 160 mL and closed with butyl rubber seals and aluminium caps. The addition of 10% v/v (9 mL) of NaHCO_3 (5g/L) solution was made to each test bottle to maintain the pH value between 7.5–7.8 [34]. The untreated or pre-treated

C. vulgaris and anaerobic inoculum were mixed to achieve a ratio of 0.5:1 on the basis of VS [27]. Quantities were calculated to obtain 90 mL of final liquid volume and to allow 43.75% of headspace. The daily biogas production was measured volumetrically, and on each measurement day, a 10 mL syringe (VWR, UK) was connected to the top of the BMP bottle to measure the daily biogas production before measuring the methane percentage, and also to make sure that the internal pressure was equal to atmospheric pressure [19]. Each BMP assay was performed in triplicate for each individual substrate in order to identify the biogas production level and percentage of methane, and a blank test containing only inoculum was also performed and subtracted from the treatment bottles. The volume of methane was calculated under standard temperature and pressure (STP) conditions (0 °C, 1atm). All BMP bottles were placed in a 37 °C incubator at 70 rpm for 25 or 26 days of reaction.

2.5. Analytical methods

TS and VS were determined according to APHA standard methods [35]. Concentrations of total COD (COD_t) and soluble COD (COD_s) were measured using Merck COD cell test kits (VWR, UK) based on the standard methods of APHA 5220 D [35]. Total and soluble carbohydrate contents were measured via the phenol-sulphuric acid method [36]. Measurements of the total and soluble protein contents were based on the bicinchoninic acid (BCA) protein assay [37]. The measurements were carried out using a protein assay kit (Thermo Scientific Pierce, UK), with bovine serum albumin (BSA) as the standard. For the measurement of COD_s , soluble carbohydrate and protein, samples were centrifuged at 3392×g for 10 minutes in a centrifuge (Sigma 3-16P, UK), and then the supernatant was filtered using a 0.2 µm nylon filter (VWR, UK).

The methane composition of the biogas was determined using a GC-FID instrument (Carlo-Erba 5160 GC) with hydrogen as the carrier gas and the injector at 150 °C and FID at 300 °C. Methane standards (10% or 80% CH_4 balanced with CO_2 ; Scientific and Technical Gases Ltd., UK) were used in triplicate injections of 50, 40, 30, 20 and 10 µL of standard gas to make a calibration standard. A minimum calibration coefficient (R^2) of 0.99 is required before the analysis of test gas samples. After that, triplicate injections of a 50 µL sample of biogas, taken from the headspace of the BMP bottles using a 100 µL gastight syringe (SGE, 100R-V-GT), were quantified by reference to the standard curve. The volume of methane was calculated under STP conditions.

2.6. Kinetics of anaerobic digestion

The modified Gompertz equation has been used in many previous studies, and also assumes that methane production is proportional to microbial activity, which indicates the growth of microorganisms [32, 38]. The kinetic data obtained from all digesters were checked for the fitness of the modified Gompertz equation using Equation 4.

$$M = P \times \exp \left\{ - \exp \left[\frac{R_m \times e}{P} (\lambda - t) + 1 \right] \right\} \quad (4)$$

where M is the cumulative methane production (ml/gVS) at time t, P is the methane yield potential (ml/gVS),

R_m is the maximum methane production rate (ml/gVS/d), λ is the duration of lag phase (d), and t is the digestion time (d).

2.7. Energy balance

To evaluate the energy balance of each pre-treatment method, the ratio between energy input (E_i) and energy output (E_o) is used as an indicator [27, 39]. The E_i was estimated using Equation 5, where the main energy input was determined by the heat required to raise the microalgal biomass from the initial temperature (T_o) to the pre-treatment temperature (T_p).

$$E_i = \frac{\rho \times V \times \gamma \times (T_p - T_o) \times (1 - \phi)}{VS} \quad (5)$$

where ρ is microalgae specific density, which was assumed to be equal to that of water of 1g/mL, V is the sample volume (mL), γ is the specific heat values of 4.18×10^3 kJ/g.°C, T_p is the pre-treatment temperatures of 37 and 55 °C for thermo-alkaline pre-treatment and, 55 and 75 °C for enzymatic pre-treatment, T_o is the initial temperature for microalgal biomass, which was assumed to be equal to the ambient temperature of 20 °C, ϕ is the heat recovery efficiency, which was assumed to be equal to 85%, and VS is the volatile solids content in pre-treated samples (g).

The energy required for pre-treatment should at least be covered by the extra methane produced; therefore, the E_o was calculated from the difference in methane yields between pre-treated and untreated microalgae using Equation 6.

$$E_o = \frac{\Delta P_{CH_4} \times LCP}{10^6} \quad (6)$$

where ΔP_{CH_4} is the enhanced methane yield after pre-treatment (mL/g VS), and LCP is the lower calorific power (LCP) of methane, which has been reported to be approximately 35.8×10^3 kJ/m³ CH₄ under standard conditions (STP) of 1atm and 0 °C [40].

2.8. Statistical analysis

For enzymatic pre-treatment, experimental data for the solubilisations of COD, carbohydrate and protein as well as methane yields were analysed by a two-way mixed analysis of variance (ANOVA) with the Bonferroni post hoc test used to determine the significance of statistical differences between the concentrations of enzymes [41]. The Turkey post hoc test was used to determine the differences between types of enzymes.

Experimental data for thermo-alkaline pre-treatment were analysed by a three-way mixed ANOVA with the Bonferroni post hoc test used to determine the statistical differences between the dosages of Ca(OH)₂ [41].

A Pearson correlation coefficient analysis was run to determine the relationship between the solubilisations of COD, carbohydrate or protein and methane yields [41]. A confidence interval of differences of 95% ($p < 0.05$) was chosen to define statistical significance. All statistical analyses were conducted using IBM SPSS statistics, Version 23. MATLAB, R2015a was used to calculate P , R_m and λ for each digester.

3. Results

3.1. Hydrolysis efficiency

Fig.1 shows the hydrolysis efficiency after the enzymatic pre-treatment. COD solubilisation after enzymatic pre-treatments ranged from 41 to 67% (Fig.1a), and the concentrations of enzymes had a significant effect on COD solubilisation ($F(1.104,30)=6500.00$, $p < 0.001$). The type of enzyme also had a significant effect on COD solubilisation ($F(4,15)=60.26$, $p < 0.001$). Enzyme mixtures showed a higher COD solubilisation than single enzymes. Cellulase plus α -amylase (CA) exhibited higher COD solubilisations, which were significantly higher than those of cellulase ($p < 0.001$), protease ($p = 0.001$) and α -amylase ($p < 0.001$). Similarly, cellulase plus protease (CP) also showed significantly higher COD solubilisation than those of cellulase ($p < 0.001$), protease ($p = 0.004$) and α -amylase ($p < 0.001$). However, COD solubilisations were not significantly different between cellulase plus protease (CP) and cellulase plus α -amylase (CA) ($p = 1.000$). The significant effect on COD solubilisation was also qualified in terms of an interaction effect between concentration and type of enzymes ($F(4.42,15)=40.14$, $p < 0.001$). This interaction effect can be seen where the COD solubilisations from single enzymes and mixed enzymes were not significantly different with an enzyme dosage at 0.5% v/w. However, the COD solubilisations observed for mixed enzymes were significantly higher than the values observed for single enzymes when the enzyme dosage was set at 1.0% v/w.

Carbohydrate solubilisation after enzymatic hydrolysis ranged from 13 to 44% (Fig.1b), and was significantly increased with increasing enzyme concentrations ($F(1.10,30)=1400.00$, $p < 0.001$). The enzyme type also significantly affected carbohydrate solubilisation ($F(4,15)=3840.00$, $p < 0.001$). For single enzymes, cellulase released significantly more carbohydrates than did protease ($p < 0.001$) and α -amylase ($p < 0.001$). Enzyme mixtures performed well compared to single enzymes in terms of carbohydrate solubilisation, where cellulase plus protease (CP) showed significantly higher carbohydrate solubilisation than cellulase ($p < 0.001$), α -amylase ($p < 0.001$) and protease ($p < 0.001$). Similarly, cellulase plus α -amylase (CA) also showed significantly higher carbohydrate solubilisation than cellulase ($p < 0.001$), α -amylase ($p < 0.001$) and protease ($p < 0.001$).

Protein solubilisation was observed to range from 28 to 72% after enzymatic pre-treatment (Figure 1c). The concentration of enzyme had a significant effect on protein solubilisation ($F(1.32,30)= 4750.00$, $p < 0.001$), and mixed enzymes exhibited significantly higher solubilisation of protein than single enzymes ($F(4,15)=657.00$, $p < 0.001$). The significant effect on protein solubilisation was also qualified by an interaction effect between concentration and type of enzymes ($F(5.29,30)=106.39$, $p < 0.001$). This interaction effect can be seen where the cellulase plus protease (CP) and single protease (P) were the most effective enzymes for protein solubilisation, and they both showed higher values than other types of enzyme.

Fig.1. Solubilisation of (a) COD, (b) carbohydrate and (c) protein after enzymatic pre-treatment. Error bar=mean $\pm$ SD, n=4.

Fig.2 shows the hydrolysis efficiency after low-temperature thermo-alkaline pre-treatment. COD solubilisation was observed to range from 18 to 28% (Fig. 2a), and $\text{Ca}(\text{OH})_2$ dosage had a significant effect on COD solubilisation ($F(3,12)=2838.00$, $p<0.001$). The highest values were obtained with a dosage of 15% w/w, and were significantly higher than those with 0% w/w dosage ($p=0.001$). However, there was no significant difference between the results with 10 and 15% w/w dosages ($p=1.000$). The pre-treatment temperature also had a significant effect on the solubilisation of COD ($F(1,4)=10.99$, $p=0.030$), with values observed at 55 °C higher than those of at 37 °C. However, pre-treatment time had no significant effect on COD solubilisation ($F(1,4)=0.00$, $p=0.983$).

Carbohydrate solubilisation was observed to range from 27 to 50% after low-temperature thermo-alkaline pre-treatment (Fig. 2b). $\text{Ca}(\text{OH})_2$ dosage had a significant effect on carbohydrate solubilisation ($F(3,12)=955.08$, $p<0.001$). The highest values were obtained with a dosage of 15% w/w, and were significantly higher than those for 0% w/w dosage ($p=0.013$). However, there was no significant difference between the results with 10 and 15% w/w dosage ($p=0.475$). Pre-treatment time and temperature both had significant effects on the solubilisation of carbohydrate ($F(1,4)=20.60$, $p=0.011$ and $F(1,4)=42.26$, $p=0.003$, respectively).

Between 14–26% of proteins were solubilized after low-temperature thermo-alkaline pre-treatment (Fig. 2c), and $\text{Ca}(\text{OH})_2$ dosage had a significant effect on protein solubilisation ($F(3,12)=1452.42$, $p<0.001$). The highest values were observed with 15% w/w dosage, and were significantly higher than those with 0% w/w ($p=0.001$) and 10% w/w dosages ($p=0.023$). The solubilisation of protein observed at 55 °C were significantly higher than those of at

37 °C ($F(1,4)=34.15$, $p=0.004$). Pre-treatment time also had a significant effect on protein solubilisation ($F(1,4)=29.28$, $p=0.006$).

Fig.2. Solubilisation of (a) COD, (b) carbohydrate and (c) protein after low-temperature thermo-alkaline pre-treatment. Error bar=mean $\pm$ SD, n=2.

3.2. Methane yields and BMP kinetic model

Fig.3 shows the cumulative amounts of methane produced by digestion of untreated and enzymatic pre-treated *C. vulgaris*. The lowest methane yield of 120 mL CH_4/g VS was produced by digestion of untreated *C. vulgaris*, and methane yields were improved by 22–162% after enzymatic pre-treatments. Methane yields were significantly improved after enzymatic pre-treatment ($F(2,50)=647.79$, $p<0.001$). The methane yields were significantly higher for the 1.0% v/w dosage than for 0.5% v/w dosage ($p<0.001$) and untreated microalgae ($p<0.001$). The type of enzyme also had a significant effect on methane yields ($F(4,25)=127.95$, $p<0.001$). The mixed enzymes showed higher methane yields compared to single enzymes, and the highest methane yields were achieved by cellulase plus protease (CP), which were significantly higher than those for cellulase ($p<0.001$), protease ($p<0.001$), α -amylase ($p<0.001$) and cellulase plus α -amylase ($p=0.002$).

Gompertz kinetics data obtained by digestion of untreated and enzymatic pre-treated *C. vulgaris* are summarized in Table 4. The values of R_m were significantly increased after enzymatic hydrolysis ($F(2,10)=166.33$, $p<0.001$). The values obtained by 0.5 and 1.0% v/w dosages were all significantly higher than for untreated *C. vulgaris* ($p<0.001$ and $p<0.001$, respectively). Enzyme type also had a significant effect on the values of R_m ($F(4,5)=11.37$, $p=0.010$). The highest R_m values were obtained by cellulase plus protease, which were significantly higher than those for cellulase plus α -amylase ($p=0.034$). However, there was no significant difference between results for cellulase plus

protease and cellulose ($p=1.000$), protease ($p=1.000$) and α -amylase ($p=0.074$). The duration of lag phase (λ) were shorter than those for untreated microalgae in some pre-treatment conditions (A1, CA1 and CA2). However, there were no significant effects of enzyme dosage and type on the time values of λ ($F(2,10)=2.56$, $p=0.127$ and $F(4,5)=3.28$, $p=0.112$, respectively).

Fig.3. Cumulative methane yields after enzymatic pre-treatment: (a) enzyme dosage at 0.5% v/w, and (b) enzymatic dosage at 1.0 % v/w.

Table 4 Summary of modified Gomerptz kinetics data for AD of untreated and enzymatic pre-treated *C. vulgaris*

No.	R_m (mLCH ₄ /gVS/d)	λ (d)	R^2
Untreated	18.88	0.70	0.9925
C1	33.32	0.96	0.9971
C2	38.82	1.14	0.9974
P1	35.61	1.06	0.9945
P2	43.17	0.95	0.9977
A1	26.31	0.58	0.9958
A2	35.92	0.91	0.9975
CP1	43.40	1.00	0.9859
CP2	36.52	0.74	0.9906
CA1	30.57	0.57	0.9885
CA2	28.69	0.44	0.9893

Fig.4 shows the cumulative methane yields after low-temperature thermo-alkaline pre-treatment. After 25 days of reaction, final methane yield of 129 mL CH₄/g VS was achieved by digestion of untreated *C. vulgaris*. The methane yields were improved by 4–19% (except for L1B) compared to untreated *C. vulgaris* when the pre-treatment temperature was 37 °C (Figs. 4a and 4b), and the highest yield of 154 mL CH₄/g VS was produced by L2B (10% w/w Ca(OH)₂ dosage, and 48 hours pre-treatment). When the pre-treatment temperature was 55 °C (Figs. 4c and 4d), the methane yields were improved by 8–26% compared to untreated *C. vulgaris*, and the highest yield of 162 mL CH₄/g VS was produced by H2A (10% w/w Ca(OH)₂ dosage, and 24 hours pre-treatment). Overall, the dosage of Ca(OH)₂ had a significant effect on

methane yield ($F(3,12)=11.34$, $p=0.001$). Pre-treatment temperature also had a significant effect on methane yield ($F(1,4)=9.79$, $p=0.035$), with yields obtained at 55 °C higher than those of at 37 °C. However, pre-treatment time had no significant effect on methane yield ($F(1,4)=0.56$, $p=0.495$).

Table 5 shows the Gompertz kinetics data of the low-temperature thermo-alkaline pre-treatment. Ca(OH)₂ dosage had no significant effect on the values of R_m ($F(3,12)=3.31$, $p=0.057$), but pre-treatment temperature had a significant effect on R_m ($F(1,4)=9.90$, $p=0.035$). Moreover, there was a significant interaction effect between Ca(OH)₂ dosage and pre-treatment temperature ($F(3,12)=9.68$, $p=0.002$). This interaction effect can be seen where at the high pre-treatment temperature of 55 °C, the values of R_m were increased compared to untreated microalgae. However, the effect of pre-treatment time on R_m was not significant ($F(1,4)=0.17$, $p=0.698$). The time values of λ were significantly increased after thermo-alkaline pre-treatment compared to untreated microalgae ($F(3,12)=57.07$, $p<0.001$). The time values of λ at 37 °C were significantly shorter than at 55 °C ($F(1,4)=159.15$, $p<0.001$). However, the values of λ did not significantly differ between 24 and 48 hours of pre-treatment time ($F(1,4)=0.92$, $p=0.392$).

Table 5 Summary of modified Gomerptz kinetics data for AD of untreated and thermo-alkaline pre-treated *C. vulgaris*

No.	R_m (mLCH ₄ /gVS/d)	λ (d)	R^2
Untreated	16.62	1.45	0.9850
L1A	9.46	1.60	0.9825
L1B	8.53	1.55	0.9868
L2A	13.85	2.35	0.9746
L2B	15.18	2.09	0.9685
L3A	17.21	2.70	0.9681
L3B	16.79	2.90	0.9793
H1A	17.20	5.96	0.9783
H1B	20.97	6.14	0.9685
H2A	22.82	2.78	0.9632
H2B	16.62	3.87	0.9833
H3A	19.66	4.17	0.9789
H3B	16.62	3.87	0.9833

3.3. Effects of hydrolysis efficiency on methane yield

A Pearson correlation was used to determine the relationship between hydrolysis efficiency (solubilisations of COD, carbohydrate and protein) and final methane yields. For enzymatic pre-treatment, there was a positive correlation between the solubilisation of COD and methane yield ($r(22)=0.755$, $p<0.001$). The solubilisation of carbohydrate also was also positively correlated with methane yield ($r(22)=0.787$, $p<0.001$). Similarly, there was a positive correlation between the solubilisation of protein and methane yield ($r(22)=0.733$, $p<0.001$).

For low-temperature thermo-alkaline pre-treatment, the solubilisations of COD and carbohydrate were both positively correlated with methane yield ($r(26)=0.476$, $p=0.014$ and $r(26)=0.457$, $p=0.019$, respectively). Moreover, there was also a positive correlation between the solubilisation of protein and methane yield ($r(26)=0.489$, $p=0.011$).

Fig.4. Cumulative methane yields after thermo-alkaline pre-treatment: (a) pre-treatment at 37 °C for 24 hours, (b) pre-treatment at 37 °C for 48 hours, (c) pre-treatment at 55 °C for 24 hours, (d) pre-treatment at 55 °C for 48 hours

3.4. Energy balance

In the current study, the methane yields were enhanced by the tested pre-treatment methods, and the energy output (E_o) and the energy input (E_i) to energy output (E_o) ratios of E_i/E_o are summarized in Tables 6 and 7. The value of E_i for enzymatic hydrolysis was estimated at 1.25 kJ/g VS, and this was calculated based on the temperature used to catalyst (55 °C) and deactivate (75 °C) the enzymes. The values of E_o increased with enzyme dosage and mixed enzymes obtained higher values than single enzymes (Table 6). The ratios of E_i/E_o were all lower than one (except A1), which indicates there were the positive energy balances for enzymatic hydrolysis.

Table 6 Methane yields, energy output (E_o) and energy ratios (E_i/E_o) of enzymatic pre-treatments

No.	Methane yield	E_o (kJ/gVS)	E_i/E_o
Untreated	120 ± 15 ^a	/	/
C1	159 ± 11	1.41	0.89
P1	169 ± 7	1.76	0.71
A1	146 ± 9	0.93	1.34 ^b
CP1	248 ± 5	4.58	0.27
CA1	213 ± 14	3.32	0.38
C2	183 ± 12	2.24	0.56
P2	194 ± 1	2.67	0.47
A2	177 ± 14	2.03	0.62
CP2	314 ± 11	6.94	0.18
CA2	291 ± 5	6.13	0.20

^a Mean ± SD, n=3;

^b The ratio of $E_i/E_o > 1$, which indicates the negative energy balance.

For low-temperature thermo-alkaline hydrolysis, values of E_i were estimated at 0.28 and 0.58 kJ/g VS for 37 and 55 °C, respectively. Higher values of E_o were achieved by high pre-treatment temperature of 55 °C compared to 37 °C. For 55 °C pre-treatment, the ratios of E_i/E_o at most conditions (except for H3A) were lower than one, whereas only L2A and L2B (10% $\text{Ca}(\text{OH})_2$ dosage) obtained positive energy balances for 37 °C pre-treatment.

Table 7 Methane yields, energy output (E_o) and energy ratios (E_i/E_o) of low-temperature thermo-alkaline pre-treatments

No.	Methane yield	E_o (kJ/gVS)	E_i/E_o
Untreated	129 ± 13 ^a	/	/
L1A	135 ± 11	0.22	1.25 ^b
L1B	127 ± 0	/	/
L2A	139 ± 6	0.37	0.77
L2B	154 ± 13	0.88	0.32
L3A	134 ± 5	0.18	1.58 ^b
L3B	136 ± 5	0.25	1.11 ^b
H1A	148 ± 5	0.67	0.86
H1B	153 ± 1	0.86	0.68
H2A	162 ± 1	1.18	0.49
H2B	154 ± 2	0.88	0.66
H3A	139 ± 6	0.35	1.66 ^b
H3B	154 ± 2	0.88	0.66

^a Mean ± SD, n=3;

^b The ratio of $E_i/E_o > 1$, which indicates the negative energy

4. Discussion

4.1. Effect of pre-treatments on hydrolysis efficiency

4.1.1 Enzymatic pre-treatment

The efficiency of enzymatic pre-treatment is linked to the composition of the microalgal cell wall [27]. In the current study, the tested microalgae strain is *C. vulgaris*, which belongs to the glucosamine-type *Chlorella* genus, and the main components of its cell wall are cellulose (25–30%) and hemicellulose (15–25%) [29–30]. Therefore, in agreement with previous studies, single cellulase at higher dosage (1.0% v/w) performed well in the solubilisation of carbohydrate compared to protease and α -amylase [25–26]. In the present study, the total protein content in *C. vulgaris* was observed at 39.2% of VS, and [11] reported that 20% of the total protein in *C. vulgaris* is bound to the cell wall. Therefore, protease could also be an optimizing enzyme for the pre-treatment of *C. vulgaris* as reported by [15, 26, 27]. In agreement with previous studies, *C. vulgaris* pre-treated by protease performed well in the solubilisations of both COD and protein. After 24 hours pre-treatment, the maximum solubilisation of protein with a 1.0% v/w dosage of protease was observed to be 72%. α -Amylase is targeted to break down the starch content in microalgae, whereas the starch content in *C. vulgaris* is generally located in the chloroplast and is composed of amylase and amylopectin together with sugars to store the energy for the cells [11]. The cell wall composition of *C. vulgaris* lacks starch, and therefore, this is likely to explain why the hydrolysis efficiency obtained by pre-treatment with α -amylase alone are lower than for cellulase and protease pre-treatments.

However, higher degrees of the solubilisations of COD and carbohydrate were observed when α -amylase was mixed with cellulase. The single enzyme may have limited hydrolytic activity, whereas mixtures of enzymes can exhibit a synergistic effect, improving the hydrolysis efficiency [42–43]. Therefore, the higher hydrolysis efficiency achieved by enzymes mixtures were probably a result of synergistic effects caused by the presence of two type of enzymes. Similarly, cellulase mixed with protease also performed well compared to single enzymes, especially in protein solubilisation. This finding corroborates the findings of [26–27]. In their studies, cellulase mixed with protease or pectinase led to higher hydrolysis efficiency compared to single enzymes.

4.1.2 Low-temperature thermo-alkaline pre-treatment

The combination of alkaline and temperature increases the surface area of cellulose due to the swelling of the biomass, and the degrees of polymerization and cellulose crystallinity are decreased [44]. In the current study, for low-temperature thermo-alkaline pre-treatment, the solubilisations of COD, carbohydrates and proteins increased significantly with $\text{Ca}(\text{OH})_2$ dosage and pre-treatment temperature. This finding agrees with those of [45] that the highest hydrolysis efficiency was observed with the highest alkali dosage and temperature.

Alkaline usually combined with high temperature (60–120 °C) to pre-treat microalgae, as reported by [6, 19, 45], and the cellulose may not be solubilized by the alkaline at low temperatures between 25 and 55 °C [21]. However, in the current study, 27–50% of the carbohydrates are solubilized, which is higher than the levels reported in previous studies by [13, 45]. In their studies, NaOH or CaO were combined with low temperatures of 25, 50 or 55 °C to pre-treat microalgae, and the solubilisation of carbohydrate ranged from 9.8–30.0%. It should be noticed that, in the current study, the highest $\text{Ca}(\text{OH})_2$ dosage was 15% w/w, which is higher than the dosages used in those previous studies, which were from 0.05 to 10% w/w. [45] found that increasing the alkaline dosage could release more of the cell wall structural sugars of glucose, xylose and arabinose at the same pre-treatment temperature. Therefore, the results of the current study indicate that increasing $\text{Ca}(\text{OH})_2$ dosage may have the potential to break down the microalgae cell wall, and solubilisation of the cell wall constituents can then occur at low pre-treatment temperatures.

From the current study, it was found that the effect of pre-treatment time had no significant effect on COD solubilisation, and this finding agrees with the results reported by [13]. Their study showed that incubation time (24 and 48 hours) barely affected COD solubilisation when using NaOH to pre-treat microalgae *C. vulgaris* and *Scenedesmus sp.*. Previous work has been reported that the contact time should be increased with decreasing temperatures when using lime for biomass pre-treatment [21]. In the current study, the pre-treatment time was set at either 24 or 48 hours, which is shorter than the in previous study of [23]. In their study, smooth cordgrass of *Spartina alterniflora* was pre-treated by lime at mild temperatures of 25–55 °C for 7–28 days. However, in comparison with the lignocellulosic biomass of cordgrass, microalgae are lignin-free substrate, and therefore the pre-treatment time may not need to be that long. Moreover, [16] found that pre-treatment temperature is the key factor that affects the sufficient hydrolysis of the microalgal cell wall rather than pre-treatment time. In their study, microalgae strain *Scenedesmus sp.* was thermally pre-treated by three temperatures of 70, 80 or 90 °C. The values of soluble COD significantly increased with temperature. These values were also significantly enhanced after 15 minutes pre-treatment, but afterwards only increased by 8% from 15 minutes to 4 hours.

4.2. Effect of pre-treatment on methane yield

In the current study, the ultimate methane yields were enhanced by both enzymatic and low-temperature thermo-alkaline pre-treatments. The improvements in methane yields were possibly proportional to levels of COD solubilisation [18]. In the current study, for both pre-treatments, COD solubilisation was positively correlated with methane yield. This probably explains why methane yields were enhanced compared to untreated *C. vulgaris*. Moreover, methane yields were also positively correlated with levels of carbohydrate and protein solubilisation. This agree with the findings of [45] that the highest solubilisation of both carbohydrate and protein resulted in the highest methane yield. For enzymatic pre-treatment, cellulase mixed with protease released more protein than other types of enzymes, and consequently

produced the highest methane yield. This result agrees with the findings of [27], who demonstrated that effective enzymatic hydrolysis should take into account proteic and polysaccharidic components. They found that the highest methane yield was produced by a protease plus esterase mix.

The rigid cell wall of microalgae protects them against attack from anaerobic microorganisms. The effective pre-treatment not only aims to improve the ultimate methane yield but also to increase methane production rate [15]. In the current study, production rates for enzymatic pre-treatment were significantly increased compared to untreated microalgae. After low-temperature thermo-alkaline pre-treatment, the production rates were also significantly increased at the high pre-treatment temperature of 55 °C. These results may suggest that the initial hydrolysis stage of AD was enhanced by the tested pre-treatment methods. The lag phase for some conditions of enzymatic pre-treatment were shorter than for untreated microalgae; however, in low-temperature thermo-alkaline pre-treatment the increased methane yields were accompanied by an extended lag phase, especially for the high concentration of 15% w/w $\text{Ca}(\text{OH})_2$. This was probably due to the generation of phenolic compounds when using a relatively high concentration of alkaline [46].

In the current study, low-temperature thermo-alkaline pre-treatment released more carbohydrates, and this may indicate that this pre-treatment is more efficient in the degradation and solubilisation of cell wall components. However, enzymatic pre-treatment released more COD and proteins than thermo-alkaline pre-treatment, and this may suggest that the microalgae cell wall is broken down more efficiently via enzymatic pre-treatment, releasing more intercellular organic matters [27]. Therefore, this likely explains why enzymatic pre-treatment produced more methane than low-temperature thermo-alkaline pre-treatment. However, [18, 47] reported that the biological decomposition of microalgal biomass via the hydrolysis of anaerobic microorganisms acts more strongly than pre-treatments. Therefore, the methane production of microalgae might be enhanced by other factors, such as the substrates to inoculum ratio, or operational conditions such as AD temperature rather than pre-treatments [39, 48]. Moreover, the high protein composition in microalgal biomass results in unbalanced carbon-to-nitrogen (C/N) ratios that may cause low methane yields [49-51].

4.3. Effect of pre-treatments on energy balance

The energy balance of microalgae pre-treatment was estimated in order to gain insights into the possibility of full-scale implementation [39]. In the current study, the extra methane production was sufficient to balance the energy required for the pre-treatment of microalgal biomass in most of tested conditions. The enzymatic pre-treatment showed the most energetically balanced conditions compared to low-temperature thermo-alkaline pre-treatment, and this result agrees with the finding of [27]. In their study, after enzymatic pre-treatment, microalgal biomass produced more methane and leading to a more positive energy balance compared to thermal and ultrasound pre-treatments.

For low-temperature thermo-alkaline pre-treatment, higher energy inputs were associated with higher pre-

treatment temperature, but more methane was also produced resulting in a more positive energy balance. This finding corroborates those of [39], who found that for microalgae after thermal pre-treatments, the E_i/E_o ratios were decreased with increasing pre-treatment temperature from 55 to 95 °C; and a positive energy balance was obtained at temperatures over 75 °C. It should be noted that, in the current study, the temperatures used of 37 or 55 °C are lower than in the previous study. However, the combination of $\text{Ca}(\text{OH})_2$ with low temperature was able to enhance methane yields leading to a positive energy balance, especially at an optimal dosage (such as 10% w/w). Therefore, the results suggest that low-temperature thermo-alkaline pre-treatment could also be an energetically efficient method in the pre-treatment of microalgae for methane production.

4.4. Implication of microalgae pre-treatment

Microalgae AD is a promising way to generate renewable energy, but the implementation of this process first requires the disruption of cell walls after harvesting microalgal biomass. Moreover, if integrating microalgae AD as a part of biorefinery process, a technique for mild cell disruption is required to ensure that cell components are released intact so that their subsequent extraction is easier [52]. In order to ensure that the pre-treatments can be used in large-scale applications, the selection of suitable pre-treatments should consider their energy demand and overall cost. In the current study, the results show that, in comparison with low-temperature thermo-alkaline pre-treatment, enzymatic pre-treatment was more energetically balanced. Moreover, in further considering the energy balance, enzymatic pre-treatment using a mixtures of enzymes may be favourable than single enzymes. Enzymatic pre-treatment is a mild cell disruption method which can break down the cell wall effectively, but the high cost of commercial enzymes could be an obstacle in applying this method at a large-scale. However, since the biofuel production process and pre-treatment efficiency are dependent on enzyme loading and, if this can be reduced, the enzyme costs will be proportionally lower [53]. Reducing the amount of enzyme consumed in hydrolysis process may reduce the enzyme loading requirement, and consequently reduce the costs [54]. The results of this study show that the AD of microalgae pre-treated with a 0.5% v/w dosage of a mixture of cellulase and protease could generate 248 mL CH_4/g VS of methane, which is higher than the values of 183 and 194 mL CH_4/g VS of methane produced by higher dosages of 1.0% v/w of cellulase and protease, respectively. Similar results were also found when using cellulase mixed with α -amylase to pre-treat microalgae. Based on these findings, enzymatic pre-treatment using mixed enzymes should consume lower amounts of enzyme compared to single enzyme pre-treatments, but should give greater methane production. Therefore, microalgae pre-treatment using mixed enzymes may be a potential way to reduce enzyme costs, and improve the economic feasibility of the pre-treatment process prior to AD.

In the current study, the microalga *C. vulgaris* pre-treated by a low-temperature thermo-alkaline method produced less methane compared to enzymatic pre-treatment. However, as discussed above, one of the requirements in the selection of a suitable pre-treatment is that it should be less

energy intensive. The results of this study also showed that a combination of 10% w/w Ca(OH)_2 with low pre-treatment temperatures of 37 and 55 °C lead to a positive energy balance. In addition, the selection of pre-treatment temperature in the current work was based on the operational temperatures used in anaerobic digesters under mesophilic (37 °C) and thermophilic (55 °C) conditions. Therefore, when considering the utilisation of the low-temperature thermo-alkaline pre-treatment of microalgae prior to AD, the pre-treatment temperature could be set at the same as the subsequent AD process. Moreover, thermo-alkaline pre-treatment using Ca(OH)_2 has additional advantages, including inexpensive reagent costs and a safety process. Therefore, a low-temperature thermo-alkaline pre-treatment of microalgae with Ca(OH)_2 as an energetically balanced and a cost-effective method, has the potential for application beyond the laboratory scale investigations.

5. Conclusion

In this study, the effect of enzymatic and low-temperature thermo-alkaline pre-treatments on microalgae solubilisation and methane yield was investigated. For enzymatic pre-treatment, the methane yields were significantly enhanced after pre-treatment, where the enzyme concentration at 1.0% v/w dosage performed the best. Moreover, mixed enzymes gave better hydrolysis efficiency and produced more methane than the use of single enzymes. For thermo-alkaline pre-treatment, the solubilisation of organic matters and methane yields increased with the pre-treatment temperature and Ca(OH)_2 dosage. However, increasing Ca(OH)_2 dosage did not significantly improve the maximum methane productions. The solubilisations of carbohydrates and proteins significantly increased with the pre-treatment time, but it had no significant effect on methane yields. In comparison with low-temperature thermo-alkaline pre-treatment, microalgae pre-treated enzymatically may release more intercellular components in terms of the concentrations of soluble COD and proteins, and these two parameters had a positive correlation with methane yields. Therefore, enzymatic pre-treatment could break down the microalgae cell wall efficiently, and producing more methane than low-temperature thermo-alkaline pre-treatment.

The results also showed that the extra methane production was sufficient to balance the energy required for the pre-treatment of microalgal biomass in most of tested conditions. Therefore, both enzymatic and low-temperature thermo-alkaline pre-treatments were considered to be energetically efficient methods to pre-treat microalgae for bioenergy production. However, the present pre-treatment work was all based on small batch BMP experiments, in order to study the performance of anaerobic microorganisms in more depth by extended feeding with pre-treated microalgal biomass, a semi-continuously fed digester is required to evaluate the benefits, or possible long-term detrimental effects of both pre-treatments in terms of energy balance and overall costs.

6. Acknowledgements

We are grateful to the Environmental Engineering Group, School of Engineering, Newcastle University, where the study was undertaken. The authors thank David Race, Amy Bell and Peter McParlin for laboratory support.

7. References

- [1] 'OECD/IEA: Energy and Climate Change', <https://www.iea.org/publications/freepublications/publication/WE02015SpecialReportonEnergyandClimateChange.pdf>, accessed 29 July 2018.
- [2] Ramanathan, V., Feng, Y.: 'Air pollution, greenhouse gases and climate change: Global and regional perspectives', *Atmospheric Environment*, 2009, 43, (1), pp. 37-50.
- [3] Ellabban, O., Abu-Rub, H., Blaabjerg, F.: 'Renewable energy resources: Current status, future prospects and their enabling technology', *Renewable and Sustainable Energy Reviews*, 2014, 39, pp. 748-764.
- [4] Dębowski, M., Zieliński, M., Kisielewska, M., Krzemieniewski, M.: 'Anaerobic co-digestion of the energy crop *Sida hermaphrodita* and microalgae biomass for enhanced biogas production', *International Journal of Environmental Research*, 2017, 11, (3), pp. 243-250.
- [5] Chinnasamy, S., Bhatnagar, A., Claxton, R., Das, K.C.: 'Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium', *Bioresource Technology*, 2010, 101, (17), pp. 6751-6760.
- [6] Harun, R., Jason, W.S.Y., Cherrington, T., Danquah, M.K.: 'Exploring alkaline pre-treatment of microalgal biomass for bioethanol production', *Applied Energy*, 2011, 88, (10), pp. 3464-3467.
- [7] Ebrahimian, A., Kariminia, H.-R., Vosoughi, M.: 'Lipid production in mixotrophic cultivation of *Chlorella vulgaris* in a mixture of primary and secondary municipal wastewater', *Renewable Energy*, 2014, 71, pp. 502-508.
- [8] Zhao, B., Ma, J., Zhao, Q., Laurens, L., Jarvis, E., Chen, S., Frear, C.: 'Efficient anaerobic digestion of whole microalgae and lipid-extracted microalgae residues for methane energy production', *Bioresource Technology*, 2014, 161, pp. 423-430.
- [9] Alaswad, A., Dassisti, M., Prescott, T., Olabi, A.G.: 'Technologies and developments of third generation biofuel production', *Renewable and Sustainable Energy Reviews*, 2015, 51, pp. 1446-1460.
- [10] Magdalena, J.A., Ballesteros, M., González-Fernandez, C.: 'Efficient anaerobic digestion of microalgae biomass: proteins as a key macromolecule', *Molecules*, 2018, 23, (5), p. 1098.

- [11] Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., Vaca-Garcia, C.: 'Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review', *Renewable and Sustainable Energy Reviews*, 2014, 35, pp. 265-278.
- [12] Demuez, M., Mahdy, A., Tomás-Pejó, E., González-Fernández, C., Ballesteros, M.: 'Enzymatic cell disruption of microalgae biomass in biorefinery processes', *Biotechnology and Bioengineering*, 2015, 112, (10), pp. 1955-1966.
- [13] Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C.: 'Autohydrolysis and alkaline pretreatment effect on *Chlorella vulgaris* and *Scenedesmus sp.* methane production', *Energy*, 2014a, 78, pp. 48-52.
- [14] Festucci-Buselli, R.A., Otoni, W.C., Joshi, C.P.: 'Structure, organization, and functions of cellulose synthase complexes in higher plants', *Brazilian Journal of Plant Physiology*, 2007, 19, (1), pp. 1-13.
- [15] Mahdy, A., Mendez, L., Blanco, S., Ballesteros, M., Gonzalez-Fernandez, C.: 'Protease cell wall degradation of *Chlorella vulgaris*: effect on methane production', *Bioresour. Technology*, 2014c, 171, pp. 421-7.
- [16] González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.P.: 'Comparison of ultrasound and thermal pretreatment of *Scenedesmus* biomass on methane production', *Bioresour. Technology*, 2012, 110, pp. 610-616.
- [17] Mendez, L., Mahdy, A., Timmers, R.A., Ballesteros, M., González-Fernández, C.: 'Enhancing methane production of *Chlorella vulgaris* via thermochemical pretreatments', *Bioresour. Technology*, 2013, 149, pp. 136-141.
- [18] Cho, S., Park, S., Seon, J., Yu, J., Lee, T.: 'Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production', *Bioresour. Technology*, 2013, 143, pp. 330-6.
- [19] Bohutskyi, P., Betenbaugh, M.J., Bouwer, E.J.: 'The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains', *Bioresour. Technology*, 2014, 155, pp. 366-372.
- [20] Feijoo, G., Soto, M., Mendez, R., Lema, J.M.: 'Sodium inhibition in the anaerobic digestion process: antagonism and adaptation phenomena', *Enzyme and Microbial Technology*, 1995, 17, (2), pp. 180-188.
- [21] Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P.: 'Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production', *Industrial & Engineering Chemistry Research*, 2009, 48, (8), pp. 3713-3729.
- [22] Torres, M.L., Lloréns, M.d.C.E.: 'Effect of alkaline pretreatment on anaerobic digestion of solid wastes', *Waste Management*, 2008, 28, (11), pp. 2229-2234.
- [23] Liang, Y.-g., Zheng, Z., Luo, X.-z., Si, Y.-b., Cao, D.-j., Nie, E., Cheng, B.: 'Lime pretreatment to improve methane production of smooth cordgrass (*Spartina alterniflora*)', *Chemical Engineering Journal*, 2013, 217, pp. 337-344.
- [24] Ilavarasi, A., Mubarakali, D., Praveenkumar, R., Baldev, E., Thajuddin, N.: 'Optimization of various growth media to freshwater microalgae for biomass production', *Biotechnology*, 2011, 10, (6), pp. 540-545.
- [25] Choi, S.P., Nguyen, M.T., Sim, S.J.: 'Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production', *Bioresour. Technology*, 2010, 101, (14), pp. 5330-5336.
- [26] Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C.: 'Enhanced methane production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* by hydrolytic enzymes addition', *Energy Conversion and Management*, 2014b, 85, pp. 551-557.
- [27] Ometto, F., Quiroga, G., Psenicka, P., Whitton, R., Jefferson, B., Villa, R.: 'Impacts of microalgae pre-treatments for improved anaerobic digestion: thermal treatment, thermal hydrolysis, ultrasound and enzymatic hydrolysis', *Water Research*, 2014, 65, pp. 350-361.
- [28] Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I.: 'Biochemical methane potential of microalgae biomass after lipid extraction', *Chemical Engineering Journal*, 2014, 243, pp. 405-410.
- [29] Abo-Shady, A.M., Mohamed, Y.A., Lasheen, T.: 'Chemical composition of the cell wall in some green algae species', *Biologia Plantarum*, 1993, 35, (4), pp. 629-632.
- [30] Yamamoto, M., Kurihara, I., Kawano, S.: 'Late type of daughter cell wall synthesis in one of the *Chlorellaceae*, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae)', *Planta*, 2005, 221, (6), pp. 766-775.
- [31] Costa, J.C., Gonçalves, P.R., Nobre, A., Alves, M.M.: 'Biomethanation potential of macroalgae *Ulva spp.* and *Gracilaria spp.* and in co-digestion with waste activated sludge', *Bioresour. Technology*, 2012, 114, pp. 320-326.
- [32] Nielfa, A., Cano, R., Fdz-Polanco, M.: 'Theoretical methane production generated by the co-digestion of organic fraction municipal solid waste and biological sludge', *Biotechnology Reports*, 2015, 5, pp. 14-21.
- [33] Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J.B.: 'Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays', *Water Science and Technology*, 2009, 59, (5), pp. 927-934.
- [34] Dechruga, S., Kantachote, D., Chaiprapat, S.: 'Effects of inoculum to substrate ratio, substrate mix ratio and inoculum source on batch co-digestion of grass and pig manure', *Bioresour. Technology*, 2013, 146, pp. 101-108.

- [35] APHA 'Standard methods for the examination of water and wastewater', in Eaton, A.D., Clesceri, L.S., Greenberg, A.E.(Ed.), (American Public Health Association, American Water Works Association, Water Environment Federation. 21st edn. 2005)
- [36] Nielsen, S.S.: 'Phenol-sulfuric acid method for total carbohydrates', in Nielsen, S.S. (Ed.): 'Food Analysis Laboratory Manual' (Boston, MA: Springer US, 2010), pp. 47-53.
- [37] Kralj, J.G., Munson, M.S., Ross, D.: 'Total protein quantitation using the bicinchoninic acid assay and gradient elution moving boundary electrophoresis', *Electrophoresis*, 2014, 35, (12-13), pp. 1887-1892.
- [38] Patil, J.H., Raj, M.A., Muralidhara, P.L., Desai, S.M., Raju, G.K.M.: 'Kinetics of anaerobic digestion of water hyacinth using poultry litter as inoculum', *International Journal of Environmental Science and Development*, 2012, 3, (2), p. 94.
- [39] Passos, F., García, J., Ferrer, I.: 'Impact of low temperature pretreatment on the anaerobic digestion of microalgal biomass', *Bioresource Technology*, 2013, 138, pp. 79-86.
- [40] Serrano, A., Siles, J.A., Chica, A.F., Martín, M.A.: 'Improvement of mesophilic anaerobic co-digestion of agri-food waste by addition of glycerol', *Journal of Environmental Management*, 2014, 140, pp. 76-82.
- [41] Field, A.: 'Discovering statistics using SPSS' (London: SAGE Publications. 3rd edn. 2009)
- [42] Yang, Q., Luo, K., Li, X.-m., Wang, D.-b., Zheng, W., Zeng, G.-m., Liu, J.-j.: 'Enhanced efficiency of biological excess sludge hydrolysis under anaerobic digestion by additional enzymes', *Bioresource Technology*, 2010, 101, (9), pp. 2924-2930.
- [43] Alvira, P., Negro, M.J., Ballesteros, M.: 'Effect of endoxylanase and α -L-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw', *Bioresource Technology*, 2011, 102, (6), pp. 4552-4558.
- [44] Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B.: 'Biomass pretreatment: fundamentals toward application', *Biotechnology Advances*, 2011, 29, (6), pp. 675-685.
- [45] Solé-Bundó, M., Carrère, H., Garfí, M., Ferrer, I.: 'Enhancement of microalgae anaerobic digestion by thermo-alkaline pretreatment with lime (CaO)', *Algal Research*, 2017, 24, pp. 199-206.
- [46] Antonopoulou, G., Dimitrellos, G., Beobide, A.S., Vayenas, D., Lyberatos, G.: 'Chemical pretreatment of sunflower straw biomass: The effect on chemical composition and structural changes', *Waste and Biomass Valorization*, 2015, 6(5), pp. 733-746.
- [47] Yen, H.-W., Brune, D.E.: 'Anaerobic co-digestion of algal sludge and waste paper to produce methane', *Bioresource Technology*, 2007, 98, (1), pp. 130-134.
- [48] Caporgno, M.P., Trobajo, R., Caiola, N., Ibáñez, C., Fabregat, A., Bengoa, C.: 'Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions', *Renewable Energy*, 2015, 75, pp. 374-380.
- [49] Schwede, S., Kowalczyk, A., Gerber, M., Span, R.: 'Anaerobic co-digestion of the marine microalga *Nannochloropsis salina* with energy crops', *Bioresource Technology*, 2013, 148, pp. 428-435.
- [50] Zhang, Y., Caldwell, G.S., Zealand, A.M., Sallis, P.J.: 'Anaerobic co-digestion of microalgae *Chlorella vulgaris* and potato processing waste: effect of mixing ratio, waste type and substrate to inoculum ratio', *Biochemical Engineering Journal*, 2019, 143, pp. 91-100.
- [51] Zhang, Y., Caldwell G.S., Sallis, P.J.: 'Semi-continuous anaerobic co-digestion of marine microalgae with potato processing waste for methane production', *Journal of Environmental Chemical Engineering*, 2019, 7(1), pp. 102917.
- [52] Vanthoor-Koopmans, M., Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M.: 'Biorefinery of microalgae for food and fuel', *Bioresource Technology*, 2013, 135, pp. 142-149.
- [53] Liu, G., Zhang, J., Bao, J.: 'Cost evaluation of cellulase enzyme for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling', *Bioprocess and Biosystems Engineering*, 2016, 39, (1), pp. 133-140.
- [54] Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B.A., Blanch, H.W.: 'The challenge of enzyme cost in the production of lignocellulosic biofuels', *Biotechnology and Bioengineering*, 2012, 109, (4), pp. 1083-1087.