Short communication

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Improving biogas production from microalgae by enzymatic pretreatment

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Abstract

In this study, enzymatic pretreatment of microalgal biomass was investigated under different conditions and evaluated using biochemical methane potential (BMP) tests. Cellulase, glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase were selected based on the microalgae cell wall composition (cellulose, hemicellulose, pectin and glycoprotein). All of them increased organic matter solubilisation, obtaining high values already after 6 hours of pretreatment with an enzyme dose of 1% for cellulase and the enzyme mix. BMP tests with pretreated microalgae showed a methane yield increase of 8 and 15% for cellulase and the enzyme mix, respectively. Prospective research should evaluate enzymatic pretreatments in continuous anaerobic reactors so as to estimate the energy balance and economic cost of the process.

Keywords:

Enzyme; hydrolysis; methane; microalgae; pretreatment; solubilisation

1. Introduction

Microalgae have been investigated in recent years for bioenergy production due to their high photosynthetic activity, ability to accumulate lipids and capacity to grow in saline, brackish and wastewaters (Park et al., 2011). Microalgal biomass may be processed for conversion into bioethanol, biodiesel and/or biogas. Nevertheless, it has been shown that biogas production through anaerobic digestion is the most straightforward technology, since neither drying nor extraction techniques are needed. Still, pretreatment methods are crucial for enhancing the hydrolysis step and increasing the methane yield due to the resistant and complex microalgae cell structure (González-Fernández et al., 2012; Passos et al., 2014).

Most microalgae cell walls are composed by two parts: a fibrillar part (skeleton) and an amorphous part (matrix). The fribillar component is formed by cellulose, mannan and xylans; while the amorphous component is where the fribillar part is submerged (Lee, 2008). Complex microalgae cell walls, such as the ones from *Chlorella* sp. and *Scenedesmus* sp., are also composed by an outer layer, which may be homogenous or have a trilaminar sheath (TLS). The TLS is resistant to the anaerobic degradation process since it is composed by sporopollenin, also called algaenan, which is a lignin-like biopolymer, formed from hydroxylated fatty acids and phenolics (Kwietniewska and Tys, 2014). Furthermore, when dealing with microalgal biomass grown in open ponds for wastewater treatment, a mixed community of microalgae and bacteria is formed. This biomass varies in terms of population dynamics, microalgae composition and cell wall structure; generally formed of a rigid cell wall, due to the variable conditions of the system, the presence of grazers and the high organic content of urban wastewater (Park et al., 2011; Passos et al., 2015a).

Thermal, mechanical and thermochemical pretreatments are among the most studied methods for improving microalgae anaerobic digestion performance (Passos et al., 2014). Such methods are used to disrupt or weaken the cell wall structure, improving macromolecules bioavailability and biodegradability in the reactor. Nevertheless, some thermal and most mechanical methods are energetically unbalanced, i.e. the energy consumed in the pretreatment step is not compensated by the biogas gain without biomass dewatering (Passos et al., 2014). In this manner, research on biogas production from microalgae should focus on technologies with low energy demand, such as biological pretreatments.

Biological methods operate with mild conditions, where microalgae cell wall is degraded enzymatically rather than disrupted as in mechanical techniques (Günerken et al., 2015). Indeed, enzymatic pretreatment consists in converting molecules from the cell wall into more usable substrates for anaerobic microorganisms. Therefore, it is necessary to know the composition of microalgae cell wall in order to select the appropriate enzymes. For most species it is composed of cellulose, hemicellulose, pectin and glycoprotein (González-Fernández et al., 2012). The hydrolysis of cellulose and hemicellulose is well studied for lignocellulosic biomass biodegradation. Celluloses are polysaccharides of glucose, more specifically they are glucose molecules linearly polymerized by β-1,4-glycosidic bonds creating cellulose chains, which are connected constituting microfibrils. Hemicelluloses are randomly branched heterogenetic polysaccharides of various mono-sugars (xylose, arabinose, galactose, mannose and rhamnose) and uronic acids (glucuronic acid, methyl glucuronic acid and galacturonic acid) (Yang et al., 2015).

Some promising results have already been shown in terms of biomass solubilisation and biogas production increase after enzymatic pretreatment of pure microalgae cultures (Ometto et al., 2014; Wieczorek et al., 2014). Nevertheless, the literature is still scarce on the effect of enzymatic pretreatment on mixed microalgal biomass grown in wastewater treatment systems. To date, results showed how the methane yield of *Chlorella vulgaris* was increased by 70% with cellulase (Onozuka) and a hemicellulose mix (Macerozyme) (Wieczorek et al., 2014). Similarly, the methane yield of the same microalgae species was increased by 86% with carbohydrolase and protease (Mahdy et al., 2014). For the filamentous microalgae *Rhizoclonium* sp., an enzyme mix composed by amylase, protease, lipase, xylanase and cellulase enhanced the methane yield by 30% (Ehimen et al., 2013).

The enzymes investigated in this study were cellulase for enhancing cellulose hydrolysis, along with glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase for enhancing hemicellulose hydrolysis. The goal was to evaluate organic matter solubilisation and methane yield increase after enzymatic pretreatment of microalgal biomass grown in open ponds for wastewater treatment.

2. Material and Methods

2.1 Microalgae-based wastewater treatment system

The experimental microalgae-based wastewater treatment system was located outdoors at the Department of Hydraulic, Maritime and Environmental Engineering of the Universitat Politècnica de Catalunya·BarcelonaTech (Barcelona, Spain). A full description of the system operation may be found in Passos et al. (2015a). Microalgal biomass was grown in a pilot high rate algal pond (HRAP) used for secondary treatment of real urban wastewater. The primary treatment consisted of a primary settler (7 L, 0.9 h hydraulic retention time (HRT)). The HRAP had a useful volume of 470 L and was operated with a HRT of 8 days. Microalgal biomass with a total solids (TS) concentration of 1.0-1.5% (w/w) was harvested in a clarifier with a useful volume of 10 L and a HRT of 4 h. Subsequently, harvested biomass was thickened in gravity-settling cones for 24 hours to increase the TS concentration before undergoing anaerobic digestion. Thickened biomass had an average composition of 4.87% TS and 3.28% volatile solids (VS) (w/w), 1.00 mg TKN (total Kjeldahl nitrogen)/L, 11.50 mg N-NH_4^+ (ammonium nitrogen)/L and pH 7.8. The macromoleular composition was 58% proteins, 22% carbohydrates and 20% lipids.

2.2 Enzymatic pretreatment

Initially, the pretreatment was carried out with the enzymes cellulase, glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase. Cellulase was provided with the commercial name of Celluclast, glucohydrolase with the commercial name of Glucanex and xylanase with the commercial name of Shearzyme by Novozymes Spain SA. For evaluating the best pretreatment conditions, two enzyme doses were compared (0.5 and 1%) over an exposure time of 6-48 hours. To this end, a volume of 100 mL of microalgal biomass was placed in Erlenmeyer flasks (150 mL) where the corresponding dose of enzyme was added (0.5 and 1% w/w). Both doses were assayed in triplicate for the three studied enzymes. Trials were set in a room with controlled temperature at 37 ºC, under continuous mixing. This temperature was set as the optimal for enzymatic activity. Samples of approximately 30 mL were removed after 6h, 12h, 24h and 48h for analysing volatile solids solubilisation after pretreatment.

2.3 Biochemical methane potential tests

Biochemical methane potential (BMP) tests were carried out for evaluating the enzymatic pretreatment effect under the best conditions selected in the former solubilisation assay. According to this, cellulase and the enzyme mix (cellulase, glucohydrolase and xylanase) were applied at a dose of 1% over an exposure time of 6 hours before undergoing BMP tests. Control trials without biomass pretreatment (microalgal biomass control) and with biomass exposed to 37 ºC for 6 hours (temperature control) were used for assessing the pretreatment effectiveness.

BMP tests were carried out in serum bottles with a total volume of 160 mL, a useful volume of 100 mL and a gas headspace volume of 60 mL. Digestate from a full-scale anaerobic reactor treating sewage sludge in a wastewater treatment plant near Barcelona (Spain) was used as inoculum. The substrate to inoculum ratio was 0.5 g VS_s/g VS_i, and each bottle contained 5 g of VS. In this case, 53 g of inoculum (18.8 g VS/L), 15 g of microalgal biomass (32.8 g VS/L) and 32 mL of distillate water were added to each bottle. Afterwards, bottles were flushed with Helium gas, sealed with butyl rubber stoppers and incubated at 35 ºC until biogas production ceased. A blank treatment was used to quantify the amount of methane produced by the inoculum. All trials were performed in triplicate.

Biogas production was determined periodically by measuring the pressure increase with an electronic manometer (Greisinger GMH 3151). After each measurement gas was released until atmospheric pressure. Samples from the gas headspace volume were taken every 2-3 days to determine biogas composition (CH_4/CO_2) by gas chromatography (GC). Results were expressed as methane yield calculated by subtracting the blank results to each trial, divided by the amount of microalgal biomass (g VS) added to each bottle.

2.4 Analytical methods

Microalgal biomass was characterised by the concentration of TS, VS, NH_4^+-N and TKN, which were measured according to Standard Methods (APHA, AWWA; WPCF, 1999); and pH, analysed with a Crison Portable 506 pH-meter. Soluble samples for VS and NH_4^+-N analyses were obtained by centrifugation (UNICEN20, 4200 rpm, 8 min, 20 ºC) and filtration (glass fiber filter 47 mm and pore size 1 µm). Microalgae identification was carried out by optic microscope examination (Axioskop 40 Zeiss, Germany), using a camera and Motic Image Plus 2.0 software and identified to genus from classical literature.

The methane content in biogas was measured with a GC (Trace GC Thermo Finnigan) equipped with a Thermal Conductivity Detector, by injecting gas samples into a packed column (Hayesep 3 m 1/8 in. 100/120). The carrier gas was Helium in split less mode (column flow: 19 mL/min). The oven temperature was 35°C with a retention time of 1.5 min. Injector and detector temperatures were 150 and 250°C, respectively. The system was calibrated with methane (50%

CH_4) and carbon dioxide (50% CO_2).

2.5 Statistical analysis

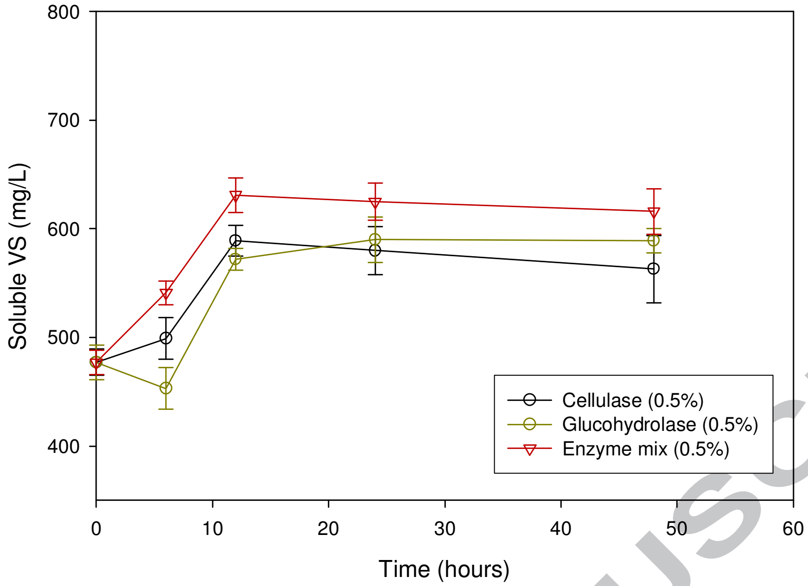
Anaerobic digestion in BMP tests was modelled by 1st order kinetics, fit by the least square method. The effect of enzymatic pretreatment on microalgal biomass solubilisation, anaerobic digestion rate and extent in BMP tests was determined by means of the ANOVA and Tukey tests; with a significance level (α) of 5%, using R Commander Statistical Software.

3. Results and Discussion

3.1 Enzymatic pretreatment

The enzymatic pretreatment was first applied for analysing microalgal biomass solubilisation. To this end, the enzymes cellulase, glucohydrolase and an enzyme mix (cellulase, glucohydrolase and xylanase) were applied at doses of 0.5 and 1% over an exposure time of 6-48 hours. The results are summarised in Figure 1. As can be seen, when an enzyme dose of 0.5% was applied, the maximum soluble VS concentration was reached after 12 h and the highest value was similar in all cases (563-616 mg VS soluble/L) (Figure 1a). For the enzyme dose of 1%, cellulase and the enzyme mix exhibited a faster solubilisation, reaching high soluble VS concentration already after 6 h of pretreatment (600 mg soluble VS/L). At the end of the experiment (48 h), both trials reached higher soluble VS (680 mg VS soluble /L) as compared to glucohydrolase (Figure 1b).

(a)



(b)

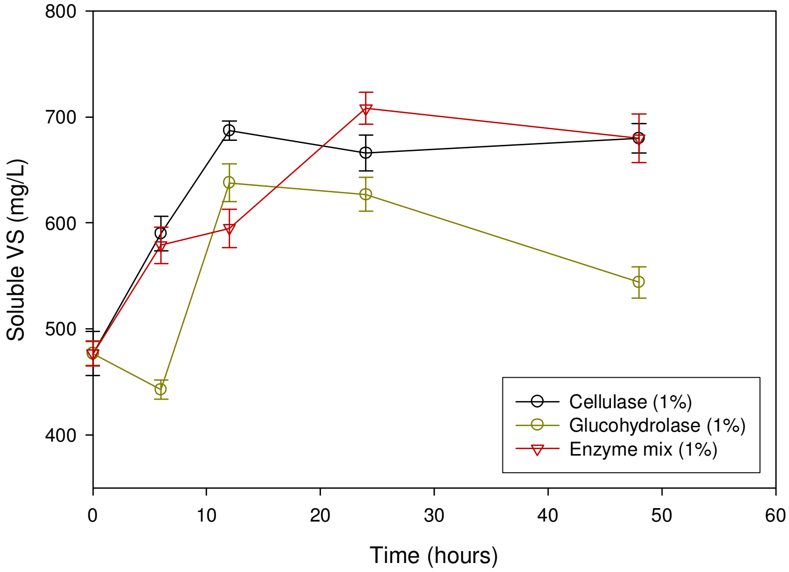


Figure 1. Volatile solids (VS) solubilisation after enzymatic pretreatment with cellulase, glucohydrolase and the enzyme mix (cellulase, glucohydrolase and xylanase) with a dose of 0.5% (a) and 1% (b).

The effectiveness of the enzymatic pretreatment is linked to the composition of microalgae cell wall. In our study, microalgal biomass consisted in a mixed community of microalgae and bacteria grown in HRAP. Generally, microalgae cells harvested from open ponds treating wastewater have a resistant cell wall due to the high organic content of the culture media and to the presence of grazers (e.g. protozoa and rotifers) (Park et al., 2011; Passos et al., 2015a). In this study, microalgal biomass was composed mainly by diatoms and *Oocystis* sp. Diatoms have a resistant nanopatterned silica layer and *Oocystis* sp. is composed by multiple external layers formed by structural polysaccharides, mainly cellulose and hemicellulose.

From the enzymes investigated, cellulase is responsible for cellulose hydrolysis, while glucohydrolase and xylanase are responsible for hemicellulose hydrolysis. The best results were reached for the enzyme mix at 0.5% dose, and cellulase and enzyme mix at 1% dose. The reason for the better performance of the enzyme mix is the synergistic effect among several macromolecules contained in the cell structure. This is to say that the enzymes glucohydrolase and xylanase may have had higher enzymatic activity after celluloses were already hydrolyzed by cellulase in the enzyme mix, i.e. in this case hemicellulose would have become more available to the enzymes. Cellulase was also effective, which may be explained by the high content of cellulose in the cell wall structure of microalgae.

These hypotheses are in agreement with the results obtained by pretreating the filamentous microalgae *Rhizoclonium* sp. with an enzyme mix of amylase, protease, lipase, xylanase and cellulase, which was more effective than applying these enzymes separately (Ehimen et al., 2013). Furthermore, the very same study also showed that cellulase accounted for the highest effect among the studied enzymes. In the case of *Chlorella vulgaris*, enzymatic pretreatment with carbohydrolase (Viscozyme) and protease (Alcalase) increased carbohydrate and protein solubilisation by 86 and 96%, respectively (Mahdy et al., 2014). Besides, fungal enzymes (*Aspergillus lentulus* and *Rhizopus oryzae*) enhanced *Chrococcus* sp. cells permeability and COD solubilisation by 29% (Prajapati et al., 2015).

3.2 Biomass solubilisation and biogas production in BMP tests

Microalgae pretreatment prior to anaerobic digestion seems imperative due to its slow biodegradability. Indeed, the methane yield reached 0.05-0.15 L CH_4/g VS in continuous reactors operated at HRT up to 20 days (González-Fernández et al., 2011). These values are low in respect to other anaerobic digestion feedstocks, such as starch and sugar crops (e.g. corn 0.18-0.41 L CH_4/g VS and potatoes 0.43 L CH_4/g VS) (Frigon and Guiot, 2010), or primary sludge (0.31 L CH_4/g VS) (Kepp and Solheim, 2000), and rather similar to waste activated sludge (WAS) (0.14 L CH_4/g VS) (Bougrier et al., 2006). Pretreatment of WAS has long been applied, although the enzymatic one has received less attention than physical and chemical methods.

In accordance with the previous section, the selected enzymatic pretreatment conditions for evaluating the anaerobic digestion performance in BMP tests were 1% of cellulase and enzyme mix for 6 hours. Results of the pretreatment and BMP tests are shown in Table 1 and Figure 2. Biomass solubilisation was increased by 110% after enzymatic pretreatment with cellulase and by 126% with the enzyme mix (Table 1). These increases were calculated by comparing the results with those obtained with the temperature control at 37ºC for 6h. Thus, the calculated solubilisation increase was only attributed to the enzymatic effect.

Table 1. Microalgal biomass solubilisation and methane yield under enzymatic pretreatment.

Trial	Soluble VS (mg/L)	Hydrolysis rate (d^{-1})	Methane yield (mL CH_4/g VS)
Microalgal biomass control	33.2 (0.6)^a	0.21 (0.004)^a	188.6 (3.2)^a
Temperature control	50.4 (3.7)^a	0.20 (0.002)^a	188.3 (0.8)^a
Cellulase	105.9 (9.1)^b	0.18 (0.002)^a	203.0 (0.4)^b
Enzyme mix (cellulase, glucohydrolase and xylanase)	114.0 (7.4)^b	0.20 (0.001)^a	217.3 (7.2)^c

^{a,b,c} Stand for significantly different values within columns (ρ = 0.05), where a refers to the lowest value and c to the highest one.

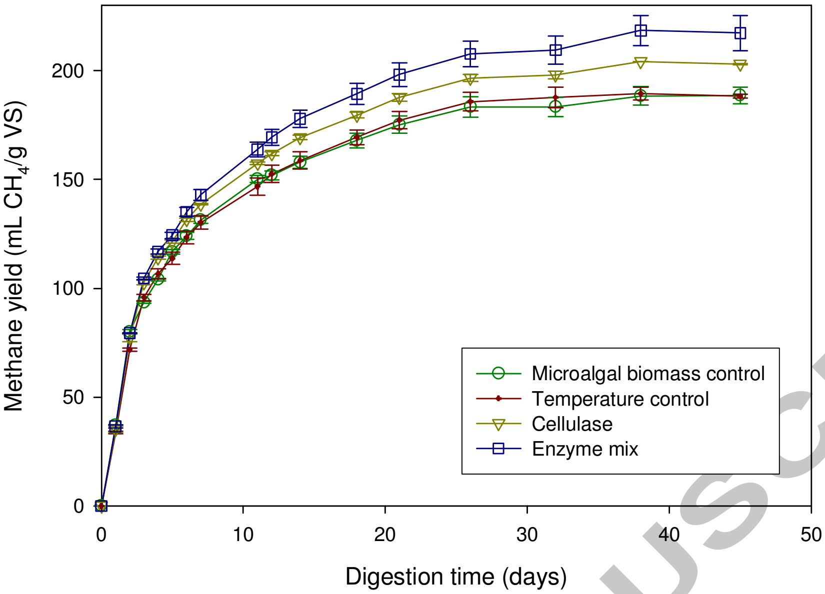


Figure 2. Accumulated methane yield in biochemical methane potential (BMP) tests under enzymatic pretreatment with cellulose and the enzyme mix (cellulase, glucohydrolase and xylanase).

The BMP test showed how the final methane yield was increased by the enzymatic pretreatment although there were no significant differences in terms of anaerobic digestion rate (Table 1). Indeed, the methane yield was significantly higher with the enzyme mix, 217 mL CH_4/g VS (15% increase), followed by cellulase, 203 mL CH_4/g VS (8 % increase), as compared to both temperature and microalgal biomass controls (188 and 189 mL CH_4/g VS, respectively). Thus, it can be concluded that there was no thermal effect of this pretreatment, but only enzymatic, and that mixing different enzymes (cellulase, glucohydrolase and xylanase) improved the performance in respect to a single enzyme (cellulase). As can be observed, the higher the VS solubilisation, the higher the methane yield in BMP tests, i.e. pretreatment with cellulase reached 110% solubilisation increase and 8% methane yield increase, while pretreatment with enzyme mix reached 126% solubilisation increase and 15% methane yield increase.

Results obtained in our study are in accordance with the literature. For instance, the anaerobic digestion of the filamentous microalgae *Rhizoclonium* sp. reached the highest methane yield after pretreatment with an enzyme mix composed by amylase, protease, lipase, xylanase and cellulase (31% increase), followed by the pretreatment with only cellulase (20% increase) (Ehimen et al., 2013). Besides, the enzymatic pretreatment of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* with carbohydrolase (Viscozyme) and protease (Alcalase) enhanced the methane yield of *C. vulgaris* by 14%, while the methane yield of *C. reinhardtii* did not increase as this species is highly biodegradable (Mahdy et al., 2014). Comparing the enzymatic pretreatment with other techniques, it was more effective than thermal hydrolysis and ultrasonication, increasing the methane yield by 270% (Ometto et al., 2014).

When comparing the results obtained with the ones found after thermal and mechanical pretreatment of microalgal biomass grown in wastewater treatment systems, values are lower. Literature results showed how the methane yield increased by 15-220% for thermal pretreatment at 70-170 °C; up to 90% for ultrasound pretreatment and up to 78% for microwave pretreatment in respect to non-pretreated biomass (Passos et al., 2014). Thermal pretreatment at low temperatures (< 100ºC) seems to be the most promising physical method so far. In fact, when comparing the effect of thermal, hydrothermal, microwave and ultrasound pretreatments on the same biomass harvested from microalgae-based wastewater treatment systems, the highest methane yield increase was achieved after thermal pretreatment at 95 °C (72%), in comparison with the other methods (8-28%) (Passos et al., 2015b). Even if the enzymatic pretreatment achieved a lower methane yield increase (8-15%), biological pretreatments have lower energy requirements compared to physical methods and, therefore, they are more likely to be compensated by the energy gain from biogas production. Furthermore, the pretreatment effect and economic cost may be improved by replacing commercial enzymes by cellulolytic bacteria and fungi from terrestrial environments. Recent studies have shown that anaerobic digestion is improved by using natural enzymes from compost, ruminant faeces or vegetable waste (Muñoz et al., 2014; Prajapati et al., 2015). In this context, prospective research should investigate the effect of enzymatic pretreatment using continuous anaerobic reactors in order to estimate the energy balance and economic cost of the process, which is yet to be determined.

4. Conclusions

Enzymatic pretreatment with cellulase and an enzyme mix composed by cellulase, glucohydrolase and xylanase were preferred to glucohydrolase due to their faster solubilisation, since cellulose was likely the main component of microalgae cell wall and hemicellulose was better hydrolysed after cellulase activity. The methane yield was significantly higher for the enzyme mix (15%) and cellulase (8%) as compared to control. Although the methane yield improvement was not as high as for physical pretreatments, it is still promising due its low energy requirement, and therefore it should be further investigated in continuous reactors to estimate the energy balance and economic cost of the process.

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Highlights

1. Enzymatic pretreatment improved microalgal biomass solubilisation and methane yield
2. The enzyme mix composed by cellulase, glucohydrolase and xylanase was most effective
3. Cellulase increased biomass solubilisation by 110% and methane yield by 8%
4. The enzyme mix increased biomass solubilisation by 126% and methane yield by 15%